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RECOMBINANT PANCREATIC LIPASES AND/OR COLIPASES AND DERIVED POLYPEPTIDES PRODUCED BY PLANTS, METHODS FOR OBTAINING SUCH AND THEIR USES

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RECOMBINANT PANCREATIC LIPASES AND/OR COLIPASES AND DERIVED POLYPEPTIDES PRODUCED BY PLANTS, METHODS FOR OBTAINING SUCH AND THEIR USES

The present invention relates to the production by plants of recombinant pancreatic lipases and colipases, in particular recombinant human pancreatic lipase (HPL) and/or human pancreatic colipase (HPCOL), in combination or individually, and other derivatives of the latter having lipase activity or possibly lipase cofactor activity, and their uses, especially as functional foods, as pharmaceutical compositions or in enzymatic formulations for agrofood or industrial applications.

Patent WO 9300426, concerning a mammalian pancreatic lipase and its variants, is known in the prior art,. The description is based on cloning and sequencing guinea pig pancreatic lipase, and the method of production described relates to expression in the filamentous fungus *Aspergillus oryzae*. However no indication is given in WO 9300426 to overcome the peculiarities and unforeseeable difficulties particular to transgenesis and to such production in plants, for example in tobacco, maize or colza.

The concerted action of pancreatic lipase and colipase enables hydrolysis of fats in the duodenum. The natural substrate of pancreatic lipase consists of long chain triglycerides dispersed in the solution of micelle bile salts. However, lipase is greatly inhibited by bile salts. When both lipase and colipase are present this inhibition is overcome. In the intestine, the pancreatic lipase-pancreatic colipase complex is formed, catalysed by long chain fatty acids which increase binding by a factor of 100. Even though lipase-colipase stoichiometry is 1:1, the lipase-colipase concentration ratio is variable according to the species under consideration (Erlanson-Albertson, 1992). In rats, the lipase-colipase ratio is less than 1 (0.48) while in man it is close to this value (1.05). This observation could be attributed to the potential role played by colipase in the regulation of the animal's weight via its activation peptide,

enterostatin.

Human pancreatic lipase (HPL) is a glycoprotein of 449 amino acids (AA) with an apparent molecular weight of approximately 50 kilodaltons (kDa) synthesised in the form of a precursor of 465 AA containing a peptide signal of 16 AA at the N-terminal side (Lowe et al 1989). Pancreatic lipases (proteins or cDNA) were purified from tissues or organs of mammals such as horse, pig, rabbit, rat or guinea pig. Between the amino acid sequences, the homology percentage is at least 80%, in particular at least 80.6% to approximately 84.6% (table 1). The homology percentage between nucleotide sequences is at least 79%, in particular at least 79.3% to approximately 87% (table 1).

Table 1

Source	% AA sequences in relation to the human sequence	% nucleotide sequences in relation to the human sequence
horse	84.6	87.0
pig	83.7	85.0
rabbit	81.2	82.8
rat	80.6	79.3

The characteristics of pancreatic lipases are the following : they are activated by lipid-water interfaces (interface activation); they are inhibited by bile salts but reactivated by colipase (activator protein); they do not significantly hydrolyse phospholipids.

Pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) plays a key part in

the absorption of food fats through the hydrolysis of triglycerides into diglycerides, then into monoglycerides and free fatty acids. Hydrolysis of triglycerides by pancreatic lipase is inhibited by physiological concentrations of bile acids. This inhibition is overcome through the addition of colipase which attaches itself to the lipase and lipid micelles. The three-dimensional structure of human pancreatic lipase has been determined by X-ray crystallography (Winkler et al 1990). It led to identifying the Ser 152 catalytic residue belonging to the Asp-His-Ser triad which is chemically analogous but structurally different from serine proteases. This catalytic site is often covered by a surface loop and is consequently inaccessible to the solvent. Interface activation, a property that is characteristic of the lipolytic enzymes acting on water insoluble substrates at water-lipid interfaces, probably requires reorientation. Human pancreatic lipase is made up of two domains, an N-terminal domain comprising residues 1 to 335, and a C-terminal domain. The N-terminal domain contains the active site, a glycosylation site (Asn 166), a Ca^{2+} fixation site, and probably a site binding intestinal heparin. The C-terminal domain contains the binding site of the colipase.

Pancreatic colipase is secreted from the pancreas in the form of a precursor, procolipase (Börgström *et al.* 1979). It contributes to the hydrolysis of the interface lipids catalysed by pancreatic lipase. Pancreatic colipase with an apparent molecular mass of 10kDa, confers catalytic activity upon pancreatic lipase under physiological conditions (high concentrations of bile salts). Procolipase is made up of 112 AA of which 17 correspond to the peptide signal. The amino acid and nucleotide sequences of human pancreatic colipase are known (Erlanson *et a*l. 1974 and Lowe *et al.* 1990).

Pancreatic colipases have been isolated from mammals. For example, rabbit pancreatic colipase has 82.7% homology of AA sequences to human colipase. Crystallographic examination of the structure of colipase and its interaction with pancreatic lipase have been studied (Egloff *et al.*, 1995). The surface of colipase can

be divided into a part that is relatively hydrophilic interacting with lipase, and a part that is more hydrophobic at the ends of the protein loops. Interaction between the colipase and the C-terminal domain of the lipase is stabilised by 8 hydrogen bonds and approximately 80 Van der Waals' contacts. When the surface loop (or "lid") covering the catalytic site releases access to the latter, 3 hydrogen bonds and approximately 28 additional Van der Waals' contacts appear, accounting for the apparent strong affinity in the presence of a lipids/water interface.

In vitro, under certain conditions, synergy of action can be shown between the gastric and pancreatic lipases on the hydrolysis of long chain triglycerides (Gargouri Y *et al.*, 1989).

Several pathological situations are known (cystic fibrosis, exocrine pancreatic deficiency) in which patients suffer from partial or total deficiency of exocrine pancreatic secretion and therefore lack the enzymes needed for food hydrolysis (amylases, lipases, proteases). Non-absorption of fats in the intestine, especially of long chain triglycerides, leads to a strong increase in steatorrhoea in such patients and, in cystic fibrosis in particular, to considerable slowing of weight gain in young patients. To correct the disorder, they are given porcine pancreatic extract at mealtimes. The therapeutic efficiency of such extracts could be considerably improved by administering HPL (due its specific action on long chain triglycerides).

Mammal cells are, in theory, more suited to the expression of mammalian genes. However, their use raises protein maturation problems. The enzymatic structure which conducts post-translational maturation varies between different tissues, organs or species. For example, it has been reported that post-translational maturation of a plasma protein may differ according to whether it is obtained from human blood or produced by a recombinant cell such as Chinese hamster ovarian cells or in the milk of transgenic animals. Also, the low expression levels obtained with mammalian cells

necessitates the use of large volumes of *in vitro* cultures at high cost. The production of recombinant proteins in the milk of transgenic animals (mice, goats, cows) can reduce production costs and overcome problems relating to expression levels. However, problems remain in respect of ethics and of viral and subviral contamination (prions).

For the above reasons, the transgenesis of mammalian genes in a plant cell could open up a means of production in great quantities of new recombinant proteins at reduced production cost and with no risk of viral or subviral contamination.

In 1983, several laboratories discovered that it is possible to transfer a heterologous gene into the genome of a plant cell and to regenerate transgenic plants from these genetically modified cells. All the plant cells then have the genetically modified character which is transmitted to subsequent generations by sexual fertilisation.

Through this work, several teams have focused their attention on the production of recombinant mammalian proteins in plant cells or in transgenic plants (Barta *et al.*, 1986). One of the first truly significant results in this area was the production of antibodies in transgenic tobacco plants.

To express a heterologous protein in grains, in which plants store their proteins, Vandekerckhove's team fused the sequence encoding leu-enkephalin with the gene coding for the albumin 2S *Arabidopsis thaliana*. With this construction, transgenic colza was produced which specifically expressed leu-enkephalin in the grains at expression levels of approximately 0.1% of total proteins. In 1990, the gene of human albumin serum was transferred into tobacco and potato cells. Irrespective of the source of the peptide signals (human or plant) levels of human albumin serum in the region of 0.02% of total proteins were obtained in the potato leaves, stalks and

tubers.

Other recombinant mammalian proteins have also been produced in plants : the surface antigen of hepatitis B, interferons, an anti-*Streptococcus mutans* mouse antibody, a caries agent, fragments of anti-cancer cell antibodies, an anti-Herpes antibody, the toxin of cholera and the epidermal growth factor (E.G.F.).

All this research has shown that the production of recombinant mammalian proteins in plant cells is possible and that the mechanisms of protein synthesis from DNA sequences are similar in animal cells and plant cells. Numerous differences exist nonetheless between plant and animal cells, in particular concerning the maturation of polymannosidic glycanns into complex glycanns, or in respect of the cleavage sites of peptide signals, which prevent the guaranteed production of active or sufficiently mammalian proteins through the transformation of plant cells.

The inventors have discovered that the use of plant cells transformed by an appropriate recombinant nucleotide sequence, can lead to obtaining pancreatic lipase, in particular recombinant HPL or proteins or polypeptides derived from the latter, having sufficient enzymatic activity for development on an industrial scale.

The purpose of the present invention is to provide a new method for obtaining, from plants, recombinant mammalian pancreatic lipases, more particularly HPL, or proteins or polypeptides derived from the latter, having enzymatic activity, more particularly lipase activity, such that said recombinant lipases, or their derived polypeptides, may be given industrial application.

Another purpose of the present invention is to provide the necessary tools for the implementation of such method, in particular new recombinant nucleotide sequences, genetically transformed plant cells, genetically transformed plants or parts

of plants (in particular leaves, stalks, fruits, seeds or grains, roots), and fragments of these genetically transformed plants or parts of plants.

A further purpose of the invention is to provide (a) new recombinant mammalian pancreatic lipase(s), in particular HPL, or any protein or derived polypeptide, that are enzymatically active, and such as obtained from genetically transformed plant cells or plants.

Another purpose of the invention is to provide new enzymatic compositions able to be used for the implementation of enzymatic reactions, especially at industrial level.

Yet another purpose of the invention is to provide new pharmaceutical compositions, in particular for the treatment of pathologies associated with insufficient lipase production in the body, such as cystic fibrosis, and for the treatment of pathologies associated with eating disorders, such as obesity.

A further purpose of the present invention is to provide new fuels, also called biofuels, having the advantage of causing less pollution than oil-derived fuels and having a lower cost price.

DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is the use of a recombinant nucleotide sequence containing firstly cDNA coding for a mammalian pancreatic lipase or for a protein or a derived polypeptide, and secondly containing elements enabling a plant cell to produce the pancreatic lipase or the protein or the derived polypeptide coded by said cDNA, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells, for the transformation of plant cells with a view to obtaining, from these cells or plants obtained from the latter, a recombinant mammalian pancreatic lipase or a protein or derived polypeptide.

In particular, the object of the present invention is the use of a recombinant nucleotide sequence containing firstly cDNA coding for any mammalian pancreatic lipase or for a protein or derived polypeptide, namely the proteins or polypeptides whose nucleotide sequences coding for the latter have *between each other* a homology percentage of at least 75%, in particular of *at least* approximately 77% to approximately 85%, whose amino acid sequences have *between each other* a homology percentage of at least approximately 75%, in particular of *at least* approximately 80% to approximately 90%, and have a lipase activity of pancreatic type, in particular a cDNA coding for any mammalian pancreatic lipase, or a cDNA coding for any protein or polypeptide derived from the above-mentioned pancreatic lipases through the addition and/or removal and/or substitution of one (or more) amino acid(s), said protein or said derived polypeptide having lipase activity of pancreatic type, and secondly containing elements enabling a plant cell to produce pancreatic lipase, or the protein or derived polypeptide, coded by said cDNA, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells, for the transformation of plant cells with a view to obtaining, from these cells, or from plants obtained from the latter, a mammalian recombinant pancreatic lipase or a protein or a derived polypeptide.

More particularly, the object of the invention is the use of a recombinant nucleotide sequence containing firstly a cDNA coding for HPL (Lowe M.E. *et al.*, 1989) or a nucleotide sequence derived from this cDNA, in particular through the addition and/or removal and/or substitution of one (or more) nucleotide(s), said derived sequence being able to code for a polypeptide whose amino acid sequence is identical to that of HPL, or for a polypeptide derived from the HPL through the addition and/or removal and/or substitution of one (or more) amino acid(s), this derived polypeptide having lipase activity of pancreatic type, and secondly containing elements enabling a plant cell to produce the polypeptide coded by said cDNA or by an above-mentioned derived sequence, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells (and more particularly by the polymerase RNAs of the latter), for the transformation of plant cells with a view to obtaining, from these cells or from plants obtained from the latter, recombinant HPL in the form of an active enzyme, or one (or more) polypeptide(s) derived from the latter such as described above.

The invention also concerns:

a recombinant nucleotide sequence characterised in that it contains firstly the sequence coding for a pancreatic lipase or a protein or a derived polypeptide, and secondly the elements enabling a plant cell to produce a pancreatic lipase or a protein or derived polypeptide coded by said sequence, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells;

-a recombinant nucleotide sequence characterised in that it contains firstly the sequence coding for a colipase or a protein or derived polypeptide, and secondly the elements enabling a plant cell to produce a pancreatic colipase or a protein or derived polypeptide coded by said sequence, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells; and

- a recombinant nucleotide sequence characterised in that it contains firstly the sequences coding for a pancreatic lipase and a colipase or the proteins or derived polypeptides, and secondly the elements enabling a plant cell to produce a pancreatic lipase and a colipase or the proteins or derived polypeptides coded by said sequence, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells.

The invention also concerns any recombinant nucleotide sequence such as described above, in particular that whose cDNA content is that of HPL, or a nucleotide sequence derived from the latter such as defined above.

Advantageously, the recombinant nucleotide sequences of the invention contain one (or more) sequence(s) coding for a peptide responsible for addressing the recombinant polypeptides of the invention (namely recombinant HPL or the above-mentioned derived polypeptides) to a determined compartment of the plant cell, particularly to the endoplasmic reticulum or the vacuoles, or even outside the cell in the pectocellulose wall or in the extracellular space also called the apoplasm.

Among the transcription terminators able to be used for the transformation of plant cells under the present invention, mention may be made of the polyA 35S terminator of the cauliflower mosaic virus (CaMV), or the polyA NOS terminator which corresponds to the 3' region non-coding for the nopalin synthase gene of the Ti plasmid of *Agrobacterium tumefaciens* nopalin strain.

In this respect, the purpose of the invention is any recombinant nucleotide sequence such as described above containing, downstream from said cDNA or its derived sequence, the polyA 35S terminator of CaMV or the polyA NOS terminator of *Agrobacterium tumefaciens*.

Among the transcription promoters able to be used for the transformation of plant cells under the present invention the following may be cited :

- the promoter 35S (P35S), or advantageously the double constitutive promoter 35S (Pd358) of CaMV, these promoters allowing the expression of the recombinant polypeptides of the invention in the entire plant obtained from the cells transformed according to the invention, and are described in the article by Kay *et al.*, 1987),

- the PCRU promoter of the gene of radish cruciferin which allows the expression of the recombinant polypeptides of the invention solely in the seeds (or grains) of the plant obtained from the cells transformed according to the invention, and described in the article by Depigny-This *et al.*, 1992,

- the PGEA1 and PGEA6 promoters corresponding to the 5' non-coding region for the genes of the reserve protein of grains, GEA1 and GEA6 respectively, of *Arabidopsis thaliana* (Gaubier *et al.*, 1993) and enabling specific expression in the grains,

- the chimerical promoter PSP super-promoter (Ni, M *et al.*, 1995), formed by fusion of triple repetition of a transcriptional activator element of the promoter of the octopine synthase gene of *Agrobacterium tumefaciens*, a transcriptional activator element of the promoter of the mannopine synthase gene, and of the mannopine synthase promoter of *Agrobacterium tumefaciens*.

- the actin promoter of rice followed by the actin intron of rice (APR-AIR) contained in the pAct1-F4 plasmid described by McElroy *et al.* (1991),

- *the HMWG (High Molecular Weight Glutenin) promoter of barley*

(Anderson O.D. *et al.*, 1989),

- the promoter of the Pyzein gene of maize (Pyzein) contained in the py63 plasmid described by Reina *et al.* (1990) allowing expression in the albumen of corn seeds.

In this respect, the object of the invention is any recombinant nucleotide sequence such as described above, containing upstream from said cDNA or its derived sequence, the double constitutive promoter 35S (Pd35S) of CaMV, or the PCRU promoter of the radish cruciferin gene, or the PGEA1 or PGEA6 promoters of *Arabidopsis thaliana*, or the PSP super-promoter of *Agrobacterium tumefaciens*, or the APR-AIR promoter of rice, the HMGW promoter of barley or the pyzein promoter of maize.

The sequences coding for an addressing peptide used within the scope of the present invention, may be of plant, human or animal origin.

Among the sequences coding for an addressing peptide of plant origin, the following may be cited :

- the nucleotide sequence of 69 nucleotides (given in the examples below) coding for the prepeptide (signal peptide) with 23 amino acids of sporamine A in sweet potato, this peptide signal allowing the entry of the recombinant polypeptides of the invention in the secretion system of the plant cells transformed in accordance with the invention (namely chiefly in the endoplasmic reticulum in particular).

- the nucleotide sequence of 42 nucleotides (given in the examples below) coding for the vacuole addressing N-terminal propeptide with 14 amino acids of sporamine A in the sweet potato, allowing accumulation of the recombinant

polypeptides of the invention in the vacuoles of the plant cells transformed according to the invention,

- the nucleotide sequence of 111 nucleotides (given in the examples below) coding for the prepropeptide with 37 amino acids of sporamine A formed, from the N-terminal side to the C-terminal side, by the 23 amino acids of the above-mentioned signal peptide followed by the 14 amino acids of the above-mentioned propeptide, this prepropeptide allowing the entry of the recombinant polypeptides of the invention in the secretion system and their accumulation in the vacuoles of the plant cells transformed in accordance with the invention, the three above-mentioned sequences being described in the articles by Murakami *et al.*, 1986 and Matsuoka *et al.*, 1991.

- the carboxyterminal propeptide of barley lectin described in particular in the articles by Schroeder *et al.*, 1993, and Bednarek *et al.*, 1991,

- and the PRS (Pathogenesis Related Protein, Cornelissen *et al.* 1986) permitting secretion.

Among the sequences coding for an addressing peptide of human or animal origin, mention may be made of that coding for the peptide signal of human pancreatic lipase (HPL) such as described by Lowe M.E. *et al.* 1989, for that of rabbit gastric lipase (RGL) such as described in European patent application EP 542629, whose sequence is given in the examples below, or for that of dog gastric lipase (DGL).

Mention may also be made, among the sequences coding for an addressing peptide, of that coding for the peptides KDEL, SEKDEL and HDEL and allowing addressing in the endoplasmic reticulum.

In this respect, the object of the invention is any recombinant nucleotide sequence such as described above, containing a sequence coding for all or part of a signal peptide such as that of sporamine A of sweet potato, or that of HPL, or that of RGL or of DGL, this sequence coding for a signal peptide being situated, in said recombinant nucleotide sequence, upstream from said cDNA or its derived sequence and downstream from the promoter used, such that the last C-terminal amino acid of the peptide signal is bound to the first N-terminal amino acid of the polypeptide coded by said cDNA or its derived sequence, in the protein coded by said recombinant nucleotide sequence.

A further object of the invention is any recombinant nucleotide sequence such as described above containing a sequence coding for all or part of a vacuole addressing peptide, in particular that of sporamine A of sweet potato, this sequence coding for a vacuole addressing peptide being situated, in said recombinant nucleotide sequence, between the sequence coding for a peptide signal and that coding for said cDNA or its derived sequence, such that the first N-terminal amino acid of the vacuole addressing peptide is bound to the last C-terminal amino acid of the peptide signal, and that the last C-terminal amino acid of said addressing peptide is bound to the first N-terminal amino acid of the polypeptide coded by said cDNA or its derived sequence, in the protein coded by said recombinant nucleotide sequence.

Another object of the invention is any recombinant nucleotide sequence such as described above containing a sequence coding for all or part of a vacuole addressing peptide, in particular that of barley lectin, this sequence coding for a vacuole addressing peptide being situated, in said recombinant nucleotide sequence, downstream from the sequence coding for said cDNA or its derived sequence, such that the first N-terminal amino acid of the vacuole addressing peptide is bound to the last C-terminal amino acid of the polypeptide coded by said cDNA or its derived sequence, in the protein coded by said recombinant nucleotide sequence.

A particular object of the invention is the following recombinant nucleotide sequences :

- that (designated Pd335-PS-HPL) containing, in 5' 3' orientation, the Pd35S promoter of CaMV, the sequence coding for the signal peptide of sporamine A, this latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated Pd35S-PPS-HPL containing, in 5' 3' orientation, the sequence coding for the prepropeptide of sporamine A, the latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated Pd35S-PSLPH-HPL) containing, in 5' 3' orientation, the Pd35S promoter of CaMV, the sequence coding for the signal peptide of HPL (orPSHPL) (namely for a sequence made up of 16 amino acids described by Lowe M.E. *et al.*, 1989), the latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated Pd35S-PSRGL-HPL) containing, in 5' 3' orientation, the Pd35S promoter of CaMV, the sequence coding for the signal peptide of rabbit gastric lipase (or PSRGL), the latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated Pd35S-PRS-HPL) containing, in 5' 3' orientation, the Pd35S promoter of CaMV, the sequence coding for the signal peptide of Pathogenesis Related Protein, the latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PCRU-PS-HPL) containing, in 5' 3' orientation, the PCRU promoter of cruciferin, the sequence coding for the signal peptide of sporamine A, the latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PCRU-PPS-HPL) containing, in 5' 3' orientation, the PCRU promoter of cruciferin, the sequence coding for the prepropeptide of sporamine A, the latter being immediately followed by the nucleotide sequence coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PCRU-PSHPL-HPL) containing, in 5' 3' orientation, the PCRU promoter of cruciferin, the sequence coding the signal peptide of HPL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PCRU – PSRGL – HPL) containing, in 5' 3' orientation, the PCRU promoter of cruciferin, the sequence coding the signal peptide of RGL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PCRU – PRS – HPL) containing, in 5' 3' orientation, the PCRU promoter of cruciferin, the sequence coding for the signal peptide of Pathogenesis Related Protein, the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PGEA1 – PSHPL – HPL) containing, in 5' 3' orientation, the PGEA1 promoter of *Arabidopsis thaliana*, the sequence encoding the signal peptide of HPL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of

CaMV,

- that (designated PGEA1 – PSRGL – HPL) containing, in 5' 3' orientation, the PGEA1 promoter of *Arabidopsis thaliana*, the sequence encoding the signal peptide of RGL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated PGEA6-PSRGL – HPL) containing, in 5' 3' orientation, the PGEA6 promoter of *Arabidopsis thaliana*, the sequence coding for the signal peptide of HPL, the latter being immediately followed by the cDNA[1] coding for mature HPL or its precursor, then the polyA 35S terminator of CaMV,

- that (designated PGEA6 – PSRGL – HPL) containing, in 5' 3' orientation, the PGEA1 promoter of *Arabidopsis thaliana*, the sequence encoding the signal peptide of RGL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV,

- that (designated APR-AIR – PSRGL – HPL) containing, in 5' 3' orientation, the APR-AIR promoter of rice, the sequence coding for the signal peptide of HPL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV, or the polyA NOS terminator of *Agrobacterium tumefaciens*,

- that (designated APR-AIR-PSRGL-HPL) containing, in 5' 3' orientation, the APR-AIR promoter of rice, the sequence coding for the signal peptide of RGL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the polyA 35S terminator of CaMV, or the polyA

NOS terminator of *Agrobacterium tumefaciens*,

- that (designated Pyzein – PSHPL – HPL) containing, in 5' 3' orientation, the pyzein promoter of maize, the sequence coding for the signal peptide of HPL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, or its precursor, then the poly 35S terminator of CaMV,

- that (designated Pyzein – PSRGL – HPL) containing, in 5' 3' orientation, the pyzein promoter of maize, the sequence coding for the signal peptide of RGL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, or its precursor, then the polyA 35s terminator of CaMV,

- that (designated Pyzein – PSHPL – HPL – KDEL) containing, in 5' 3' orientation, the pyzein promoter of maize, the sequence coding for the signal peptide of HPL (such as described above), the latter being immediately followed by the cDNA coding for mature HPL, then the sequence coding for the KDEL tetrapeptide, then the poly 35S terminator of CaMV.

Advantageously, the recombinant nucleotide sequences of the invention also contain a nucleotide sequence that can be used to label said recombinant sequences, in particular to differentiate (and therefore select) those plant cells which are transformed by said recombinant sequences from those which are not.

Preferably, such nucleotide sequence which can be used to label said recombinant sequences, is chosen from among the antibiotic resistance genes, in particular the kanamycine resistant gene.

A further object of the invention is any vector, especially plasmid vector,

containing a recombinant nucleotide sequence of the invention, inserted at a site that is non-essential for its replication.

The invention also concerns any host cell, in particular any bacterium such as *Agrobacterium tumefaciens*, transformed by a vector such as defined above.

The invention also concerns a method of obtaining recombinant pancreatic lipase, or a protein or a derived polypeptide, characterised in that it comprises :

- transforming plant cells, in particular using a host cell of the invention, itself transformed by a vector of the invention, such that a recombinant sequence of the invention is incorporated in the genome of these cells,

- if required, obtaining transformed plants from the above-mentioned transformed cells,

- recovering recombinant pancreatic lipase or the protein or derived polypeptide produced in said above-mentioned cells or transformed plants, in particular by extraction, optionally followed by purification.

For example, the object of the present invention is any method for obtaining a recombinant human pancreatic lipase in the form of an active enzyme and/or one (or more) polypeptide(s) derived from the latter, in particular through the addition and/or removal and/or substitution of one (or more) amino acid(s), this (or these) derived polypeptide(s) having lipase activity, characterised in that it comprises :

- the transformation of plant cells such as to incorporate, in the genome of these cells, one (or more) recombinant nucleotide sequence(s) of the invention,

- if required, the production of transformed plants from the above-mentioned transformed cells,

- recovery of recombinant human pancreatic lipase and/or of the above-mentioned derived polypeptide(s) produced in said above-mentioned cells or transformed plants, in particular by extraction, optionally followed by purification.

According to one embodiment of the above-mentioned method of the invention, the transformation of plant cells may be made by transferring the recombinant nucleotide sequence of the invention to the protoplasts, in particular after incubation of the latter in a polyethylene glycol solution (PEG) in the presence of divalent cations (Ca^{2+}).

Transformation of the plant cells may also be conducted by electroporation.

Transformation of plant cells may also be made using a gene gun which sprays metal particles coated with recombinant nucleotide sequences of the invention at very high speed, thereby delivering genes inside the cell nucleus.

Another method of transforming plant cells is cytoplasmic or nuclear micro-injection.

According to one particularly preferred embodiment of the above-mentioned method of the invention, the plant cells are transformed by placing them in contact with a host cell transformed by a vector of the invention, such as described above, said host cell being able to infect said plant cells by allowing the incorporation in the genome of the latter, of the recombinant nucleotide sequences of the invention initially contained in the genome of the above-mentioned vector.

Advantageously, the above-mentioned host cell is *Agrobacterium tumefaciens*, particularly in accordance with the methods described in the articles by Bevan, 1984 and An *et al.*, 1986, or *Agrobacterium rhizogenes*, in particular according to the method described in the article by Jouanin *et al.*, 1987.

Among the plant cells able to be transformed under the present invention, mention may be made of colza, tobacco, maize, pea, tomato, carrot, wheat, barley, potato, soybean, sunflower, lettuce, rice and alfalfa.

According to one embodiment of the above-mentioned method of the invention, the plant cells transformed according to the invention, are cultured *in vitro*, in particular in bioreactors in a liquid medium or in immobilised form, or by *in vitro* culture of transformed roots.

The above-mentioned *in vitro* culture media are then recovered to extract therefrom, and if necessary purify, in particular by chromatography, the pancreatic lipase, in particular recombinant HPL and/or the proteins or derived polypeptides defined above produced by said transformed cells cultured *in vitro*.

According to one preferred embodiment of the above-mentioned method of the invention to obtain pancreatic lipase, in particular recombinant HPL and or the proteins or derived polypeptides of the invention, the transformation of plant cells is followed by a production stage of transformed plants by placing said transformed cells in an appropriate culture medium. Pancreatic lipase, in particular HPL and/or the proteins or derived polypeptides produced in the cells of the entire plants obtained in this way are collected by extraction made from the entire plants or parts of these plants (in particular from the leaves, stalks or fruits) or from seeds derived from these plants, extraction possibly being followed by a pancreatic lipase purification stage, in particular of recombinant HPL and/or of the protein or the derived polypeptide(s).

The transformed plants used to recover pancreatic lipase, in particular the recombinant HPL and/or the protein(s) or derived polypeptide(s) following the above-mentioned method, are those of generation T0, namely those obtained from cultures of transformed cells of the invention on an appropriate medium, or advantageously those of the following generations (T1, T2 etc.) obtained for example by self-fertilisation of the plants of the previous generation and in which the recombinant nucleotide sequences of the invention reproduce themselves according to Mendel's law.

The invention targets more particularly a method of obtaining recombinant HPL, such as described above, characterised in that it entails :

- transforming the cells of explants from plant leaves, by placing the latter in contact with an *Agrobacterium tumefaciens* strain transformed by a plasmid such as described above containing the recombinant nucleotide sequence chosen from the following list : Pd35S-PS-HPL, Pd35S-PPS-HPL, Pd35S-PSRGL-HPL and Pd35S-PSHPL-HPL, mentioned above, on an appropriate culture medium.

- selecting explants transformed on a medium containing kanamycine,

- obtaining transformed plants from the above-mentioned transformed explants by culture of the latter on appropriate media,

- extracting the recombinant HPL in particular by grinding the leaves and/or grains and/or fruits of the above-mentioned transformed plants in an appropriate buffer, followed by centrifugation and collection of the supernatant which forms the plant extract having enzymatic activity,

- possibly purifying the recombinant HPL from the extract obtained during the previous stage, in particular by chromatography made using the

supernatant, which leads to obtaining recombinant HPL in essentially pure form.

The plant cells transformed under the above-described methods are advantageously chosen from among tobacco, colza, maize, pea, tomato, carrot, wheat, barley, potato, soybean, sunflower, lettuce, rice and alfalfa.

The invention targets more particularly a method of obtaining recombinant HPL, characterised in that it comprises :

- transforming the cells of maize calli, using a particle gun to bombard the latter with plasmids containing the recombinant nucleotide sequence APR-AIR-PSHPL-HPL and/or the sequence Pyzein-PSRGL-HPL and/or the sequence Pyzein-PSHPL-HPL-KDEL,

- selecting the transformed calli on a medium containing a selective agent such as kanamycine,

- obtaining transformed maize plants from the above-mentioned transformed calli by placing the latter in culture on appropriate media,

- extracting the recombinant HPL, in particular by grinding, in an appropriate buffer, from the seeds derived from the above-mentioned transformed plants, followed by centrifugation and recovery of the supernatant forming the plant extract having enzymatic activity,

- if needed, purifying the HPL using the extract obtained during the preceding stage, in particular by chromatography using the supernatant, which leads to obtaining HPL in essentially pure form.

A further object of the invention is any genetically transformed plant cell, containing one (or more) recombinant nucleotide sequence(s) of the invention incorporated in stable manner in their genome.

The invention also concerns any transgenic plant cell such as described above containing one (or more) recombinant protein(s) or polypeptide(s) of the invention, such as HPL, said plant cell also being termed an enzymatically active plant cell, and more particularly having lipase activity such as defined below.

A further object of the invention is genetically transformed seeds containing one (or more) recombinant nucleotide sequences such as described above, according to the invention, incorporated in stable manner in their genome.

The invention also concerns the above-described transgenic seeds, containing one (or more) recombinant protein(s) or polypeptide(s) of the invention such as $HPL^{recombinant}$, said seeds also being termed enzymatically active seeds, and more particularly having lipase activity defined below.

The transformed seeds of the invention are those harvested from genetically transformed plants of the invention, these transformed plants being either those of the above-mentioned T0 generation obtained by culture of the transformed cells of the invention, or those of the following generations (T1, T2 etc..) obtained for example by self-fertilisation or cross-breeding of the plants of previous generations (as indicated above).

A further object of the invention concerns the plants or parts of plants (in particular explants, stalks, leaves, fruits, roots, seeds, pollen, etc..) that are genetically transformed, characterised in that they contain one (or more) recombinant nucleotide sequence(s) of the invention, incorporated in stable manner in their genome.

The invention also concerns the transgenic plants or parts of plants described above, containing one (or more) recombinant protein(s) or polypeptide(s) of the invention, such as HPL, said plants or parts of plants also being termed enzymatically active plants or parts of plants, and more particularly having lipase activity such as defined below.

One particular object of the invention concerns the above-mentioned transformed plants, such as obtained by culture of the cells or seeds such as described above in accordance with the invention.

The transformed plants, or parts of plants, of the invention are advantageously chosen from among colza, tobacco, maize, pea, tomato, carrot, wheat, barley, potato, soybean, sunflower, rice, lettuce, alfalfa and beetroot, or parts of these plants.

The object of the present invention is any plant extract having enzymatic activity, for example lipase activity as defined below, such as obtained by implementing one of the above-mentioned methods of the invention, which contains recombinant pancreatic lipase and/or recombinant colipase or the proteins or derived polypeptides.

The lipase (or lipolytic) activity of the plants or parts of plants and of the enzymatically active plant extracts of the invention, may be measured using in particular the method described by Duan R *et al.*, 1991, using a short chain triglyceride (such as tributyrine) as substrate, or the method described by Egloff M.P. *et al.*, 1995. Enzymatic activity is recorded in U units, one U unit corresponding to the quantity of enzyme required to release one µmole of free fatty acids per minute at 37 C under optimal pH conditions.

The enzymatically active plant extracts of the invention are advantageously

such that the weight percentage of enzymatically active recombinant polypeptides represents approximately 0.1% to 20%, in particular approximately 1% to approximately 15%, in relation to the total weight of the proteins present in these extracts.

A further, more particular object of the invention, concerns the following enzymatically active plant extracts :

- extracts of leaves and/or fruits and/or plant grains, such as obtained by transformation of explant cells of these plants with the sequence Pd35S-PSHPL-HPL, or the sequence Pd35S-PS-HPL, or the sequence Pd35s-PPS-HPL, or the sequence Pd35S-PSRGL-HPL according to one of the above-described methods, and containing recombinant HPL,

- extract of tobacco leaf such as obtained by transforming explant cells of tobacco leaves with the sequence Pd35S-PS-HPL, or the sequence Pd35S-PPS-HPL according to the above-described method,

- extract of tobacco leaf such as obtained by transforming explant cells of tobacco leaves with the sequence Pd35S-PSRGL-HPL or the sequence Pd35S-PSHPL-HPL according to the above-described method,

- extract of tobacco grains, such as obtained by transforming the explant cells of tobacco leaves with the sequence Pd35S-PS-HPL, or the sequence Pd35S-PS-HPL or the sequence Pd35S-PPS-HPL in accordance with the above-described method,

- extracts of plant grains such as obtained by transforming explant cells of these plants with the sequence PCRU-PS-HPL, or the sequence PCRU-PPS-HPL,

or the sequence PGEA1-PSRGL-HPL, or the sequence PGEA6-PSHPL – HPL in accordance with one of the above-described methods, and containing the recombinant HPL,

- extract of colza grains, such as obtained by transforming the explant cells of colza leaves with the sequence PCRU-PS-HPL, or the sequence PCRU-PPS-HPL, or the sequence PGEA1-PSHPL-HPL, or the sequence PGEA6-PSHPL-HPL in accordance with the above-described method,

- extracts of plant seeds such as obtained by transforming the explant cells of these plants with the sequence APR-AIR-PSHPL-HPL and/or the sequence Pyzein-PSHPL-HPL and/or the sequence Pyzein-PSHPL-HPL-KDEL, in accordance with one of the above-described methods, and containing the recombinant HPL,

- extract of maize seeds, such as obtained by transforming maize seeds (in particular maize calli) with the sequence APR-AIR-PSHPL-HPL and/or the sequence Pyzein-PSRGL-HPL and/or the sequence Pyzein –PSHPL-HPL-KDEL in accordance with the above-described method.

The object of the present invention is a recombinant pancreatic lipase or protein or derived polypeptide, characterised in that it is obtained using the method of the invention.

One particular object of the invention is any enzymatically active recombinant pancreatic lipase, in particular HPL, or proteins or derived polypeptides, in particular through the addition and/or removal and/or substitution of one (or more) amino acid(s), these derived polypeptides having lipase activity, such as obtained in essentially pure form by applying one of the above-described methods of the invention, these methods comprising a purification stage of the recombinant

polypeptides of the invention, in particular by chromatography made using the enzymatic extracts described above.

By enzymatically active recombinant pancreatic lipase, or derived polypeptides having lipase activity, according to the invention, is meant any recombinant polypeptide able to have lipase activity of pancreatic type, such as for example that measured using the method described by Duan or by Egloff.

The invention more particularly concerns the recombinant HPL such as obtained by purification of the enzymatic extract of tobacco leaves or grains, these leaves or grains being derived from transformed tobacco plants, themselves obtained from tobacco cells transformed with the sequence Pd35S-PSRGL-LPH or the sequence Pd35S-PSHPL-HPL in accordance with the above-described method, said recombinant HPL having a lipase activity such as described above.

The invention concerns the antibodies directed against the recombinant polypeptides of the invention, and more particularly those directed against the recombinant HPL of the invention.

Such antibodies may be obtained by immunising an animal with these polypeptides, followed by recovery of the antibodies formed.

Needless to say, this production is not restricted to polyclonal antibodies.

It also applies to any monocloncal antibody produced by any hybridome able to be formed, by conventional methods, from the spleen cells of an animal, mouse or rat in particular, immunised against one of the purified polypeptides of the invention, and also from the cells of an appropriate myeloma, and able to be selected through its capacity to produce monoclonal antibodies which recognise the above-mentioned

polypeptide initially used to immunise the animals.

The invention also concerns the use of plants, parts of plants, plant cells, or seeds transformed in accordance with the invention, to obtain one (or more) recombinant proteins or polypeptides of the invention, such as recombinant HPL, or its derived polypeptides such as defined above, in particular by using one of the above-mentioned methods of the invention, said recombinant polypeptides being in essentially pure form or contained in enzymatically active plant extracts such as defined above.

The invention concerns in particular :

- the use of plants *or parts* of plants of the invention and/or plant extracts of the invention, and/or proteins or polypeptides or an association of the latter, in accordance with the invention, to obtain medicinal products intended to treat pathologies associated with deficient lipase production in the body, such as cystic fibrosis, or for the treatment of eating disorders, such as obesity.

- the use of plants *or parts* of plants of the invention, and/or plant extracts of the invention, and/or proteins or polypeptides or an association of the latter in accordance with the invention, to obtain foods *intended for human or animal nutrition*, in particular functional foods more particularly intended to facilitate the absorption of animal or vegetable fats ingested by healthy persons or those suffering from one or more pathologies which may or may not affect the production level of gastric and/or pancreatic lipase.

- and the use of plants *or parts* of plants of the invention, and/or plant extracts of the invention, and/or proteins or polypeptides or an association thereof, in accordance with the invention, to implement enzymatic reactions in the industrial,

agro-food or agro-industrial sectors, in particular in the fat and lipochemistry industries and the dairy industry.

The invention more particularly concerns the use as foods of plants, or parts of plants, in particular leaves, fruits, seeds with enzymatic activity according to the invention.

In this respect, one particular object of the invention is any functional food made up of an enzymatically active plant such as described above, or parts of this plant, in particular the leaves or fruits or even the seeds derived from the latter, and likely to be edible for Man or animal.

The invention also targets any functional food containing one (or more) enzymatically active plant(s) such as described above, and/or parts of this (these) plant(s), in particular the leaves and/or seeds and/or fruits of this (these) plant(s), and/or one (or more)plant extract(s) having enzymatic activity such as described above, and/or one (or more) recombinant proteins or polypeptides of the invention, possibly in association with one (or more) other edible compound(s).

For example, one particular object of the invention is any above-mentioned food composition containing recombinant HPL obtained according to the invention in essentially pure or partly pure form or in plant form and/or enzyme extract form such as described above, possibly associated with one or more other enzymatic activities helpful for digestion, preferably an enzymatic activity of amylase, protease type (in particular trypsine and chymotrypsine and/or elastase).

Advantageously, the plants or parts of plants contained in the above-mentioned food composition are in the form of grindings.

The foods of the invention, also called functional foods, or the food compositions of the invention are more particularly intended to facilitate the absorption of fat or vegetable fats ingested by healthy persons or those suffering from one or more pathologies which may or may not affect the production level of gastric and/or pancreatic lipase. In this respect, the foods or food compositions of the invention are advantageously used as nutritional supplements.

Another object of the invention is the use of plants, or parts of plants, in particular leaves and/or fruits and/or seeds, or enzymatically active plant cells of the invention, or enzymatically active plant extracts such as defined above, or recombinant polypeptides of the invention, such as recombinant HPL, or its derived polypeptides such as defined above, to obtain medicinal products (or pharmaceutical compositions) intended to facilitate the absorption of animal or vegetable fats ingested by healthy persons or those suffering from one or more pathologies which may or may not affect the production level of gastric and/or pancreatic lipase, or to correct an eating disorder such as obesity.

In particular, said pharmaceutical compositions are advantageously used in persons undergoing medical treatment which impairs the fat absorption mechanism, or in elderly persons.

The pharmaceutical compositions of the invention are also more particularly intended for the treatment of pathologies related to lipase deficiency (in particular gastric and/or pancreatic lipase(s)) in the body, and more particularly for pathologies such as cystic fibrosis, exocrine pancreatic deficiency, and obesity.

A particular object of the invention is any pharmaceutical composition, characterised in that it comprises plants *or parts* of plants according to the invention, and/or plant extracts of the invention, and/or proteins or polypeptides or an

association thereof in accordance with the invention, possibly in association with one or more pharmaceutical vehicles or excipients.

A further particular object of the invention is any above-mentioned pharmaceutical composition containing recombinant HPL in essentially pure form or in the form of enzymatic extracts such as described above.

For example, a particular object of the invention is any above-mentioned pharmaceutical composition containing recombinant HPL in essentially pure or partly pure form, or in the form of enzymatic extracts such as defined above, associated with one or more enzymatic activities helpful for digestion, preferably an enzymatic activity of amylase, protease type (in particular trypsine and chymotrypsine and/or elastase).

The pharmaceutical compositions of the invention can preferably be administered by oral route and are in the form of capsules, tablets or dissolvable powders.

The daily dosage in man is advantageously approximately 200 mg to 1000 mg, preferably divided into doses to be taken at main mealtimes, when said pharmaceutical compositions contain enzymatic extracts such as defined above, and approximately 100 mg to approximately 500 mg when said pharmaceutical compositions contain recombinant polypeptides of the invention in essentially pure form.

Another object of the invention is the use of plants, or parts of plants, in particular leaves and/or fruits and/or seeds, or of enzymatically active plant cells of the invention, or of enzymatically active plant extracts such as defined above, or of recombinant polypeptides of the invention, such as recombinant HPL, or its derived

polypeptides such as defined above, to implement enzymatic reactions in industrial, agro-food or agro-industrial areas, in particular in the fat and lipochemistry industries and the dairy industry.

In this respect, the invention concerns any method, in particular enzymatic bioconversion or biocatalysis, which uses one or more enzymatic reactions in industrial, agro-food or agro-industrial areas, in particular the fat and lipochemistry industries and the dairy industry, these enzymatic reactions being conducted by means of plants, or parts of plants, in particular leaves and/or fruits and/or seeds, or of enzymatically active plant cells of the invention, or of enzymatically active plant extracts such as defined above, or of recombinant polypeptides of the invention, such as recombinant HPL, or its derived polypeptides such as defined above.

One more particular object of the invention concerns enzymatic preparations intended for industrial, agro-food or agro-industrial use, able to be used for the implementation of a method such as described above, comprising plants, *or parts* of plants according to the invention, and/or plant extracts of the invention, and/or proteins or polypeptides or an association of the latter in accordance with the invention, possibly in association with one (or more) additives or other enzyme(s) able to be used under the above-mentioned industrial application.

The invention more particularly concerns the use of plants, or parts of plants, in particular leaves and/or fruits and/or seeds, or the use of enzymatically active plant cells of the invention, for the implementation, at industrial level, of enzymatic bioconversion or biocatalysis reactions, such as hydrolyses or enzymatic trans-esterification.

Advantageously, the plants having enzymatic activity, or parts of these plants, in particular the leaves and/or fruits and/or seeds, or plant cells of the invention, are

used both as an enzymatic source and as a reaction substrate.

Another object of the invention is any biocatalysis method using plants, or parts of plants, in particular leaves and/or fruits and/or *seeds or plant cells*, having enzymatic activity according to the invention, and more particularly plants containing HPL, said plants or parts of plants being used both as an enzymatic source and as a reaction substrate.

The invention more particularly concerns the use of enzymatically active plants, or parts of these plants, according to the invention, to obtain biofuels.

In this respect, the object of the present invention is any method for obtaining a biofuel by addition of alcohol, in particular methanol or ethanol, to the grindings of plants or parts of plants transformed according to the invention, advantageously grindings of colza, sunflower or soybean grains, transformed in accordance with the invention, and recovery of the biofuel, in particular by filtration.

The invention also concerns the esters of plant fatty acids such as those obtained by applying the above-mentioned method, in particular the methyl ester of oleic acid.

Another object of the invention is any biofuel such as obtained by applying a method such as described above, and more particularly any above-mentioned biofuel comprising esters of plant fatty acids.

The invention particularly targets any biofuel such as obtained by implementing the above-mentioned method using colza grains and comprising a methylester of oleic acid.

The invention also targets the use of the above-mentioned antibodies directed against the recombinant polypeptides of the invention, for the implementation of a detection or titration method for HPL in a biological sample likely to contain such.

The invention particularly concerns the use of these antibodies to implement an *in vitro* diagnosis method for pathologies related to overproduction, or conversely, to deficient or even lack of lipase production in the body.

This *in vitro* diagnosis method, which uses a biological sample taken from a patient, comprises a contact stage of this sample with one or more antibodies of the invention, followed by a detection stage of any HPL-antibody complexes produced during the preceding stage.

In this respect, the invention also concerns a kit for the application of an *in vitro* detection or diagnosis method as mentioned above, comprising :

- antibodies such as described above, advantageously with radioactive or enzymatic labelling, and reagents to form a medium suitable for conducting the immunology reaction between these antibodies and the HPL,

- reagents allowing the detection of immunological complexes formed between these antibodies and the HPL.

The invention also concerns the association of pancreatic-type lipase activity, obtained according to the invention, with colipase-type activity, and use of the resulting product of this association in particular for the treatment of pathologies related to deficient lipase production in the body, such as cystic fibrosis, or for the treatment of eating disorders, such as obesity.

The colipase-type activity may be derived from plant extracts expressing a recombinant colipase (for example human colipase) or a derivative of this colipase, a derivative being any protein or polypeptide derived from the above-mentioned colipase through the addition and/or removal and/or substitution of one (or more) amino acid(s), this protein or this derived polypeptide having colipase-type activity. More particularly, the colipase-type activity may be derived from a colipase or a derivative of the latter that is purified or partly purified from plants or plant extracts expressing a recombinant colipase (for example human pancreatic colipase) or a derivative of this colipase. The activity of colipase type, for example human colipase, may therefore be produced from a transgenic plant according to the above-described methods for the production of pancreatic lipase. The colipase may in particular be co-expressed in the same transgenic plant as the pancreatic lipase. It may also be expressed in a different plant and subsequently associated. The colipase-type activity may also be derived from extracts of animal origin.

The invention therefore also concerns, possibly in similar manner to that described for the pancreatic lipase activity of the invention :

use of a recombinant nucleotide sequence containing firstly a cDNA coding for a mammalian colipase or for a protein or a derived polypeptide, and secondly the elements enabling a plant cell to produce the colipase, or the protein or the derived polypeptide, coded by said cDNA, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells, for the transformation of plant cells with a view to obtaining, from these cells, or from the plants obtained from the latter, a mammalian recombinant colipase, or a protein or a derived polypeptide,

use of sequences for the co-transformation of plant cells with a view to obtaining, from these cells, or from plants obtained from the latter, a recombinant

mammalian lipase and co-lipase, or their derivatives,

- the recombinant nucleotide sequence, characterised in that it contains firstly the sequence coding for a colipase or a protein or derived polypeptide, and secondly the elements enabling a plant cell to produce a pancreatic colipase or a protein or derived polypeptide coded by said sequence, in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells,

- the recombinant nucleotide sequence, characterised in that it contains firstly the sequences coding for a pancreatic lipase and a colipase or the proteins or derived polypeptides, and secondly the elements enabling a plant cell to produce a pancreatic lipase and a colipase or the proteins or derived polypeptides coded by said sequence in particular a promoter and a transcription terminator recognised by the transcriptional machinery of the plant cells,

- a vector, in particular a plasmid, containing a nucleotide sequence such as defined above, inserted at a site that is non-essential for its replication,

- a host cell, in particular any bacterium such as *Agrobacterium tumefaciens*, transformed by a vector such as defined above,

- a method for obtaining a recombinant colipase, or a protein or a derived polypeptide, characterised in that it comprises :

- the transformation of plant cells, in particular using a host cell of the invention, itself transformed by a vector of the invention, such as to integrate into the genome of these cells a recombinant sequence of the invention,

- optionally, obtaining transformed plants from the above-mentioned

transformed cells,

- recovery of the recombinant colipase, or the protein or derived polypeptide produced in said above-mentioned transformed cells or plants, in particular by extraction possibly followed by purification,

- a method for co-obtaining a recombinant pancreatic lipase and colipase, or protein or derived polypeptides, characterised in that it comprises :

- the transformation of plant cells, in particular using a host cell of the invention, itself transformed by a vector of the invention such as to integrate into the genome of these cells a recombinant sequence of the invention,

- optionally obtaining transformed plants from the above-mentioned transformed cells,

- recovery of the recombinant pancreatic lipase and colipase or proteins or derived polypeptides produced in said above-mentioned transformed cells or plants, in particular by extraction, possibly followed by purification,

- the plants, *or parts* of plants, *in particular the leaves and/or fruits and/or seeds and/or* plant cells, genetically transformed, characterised in that they contain one (or more) recombinant nucleotide sequence(s) of the invention integrated in stable manner in their genome, these plants being chosen in particular from among colza, tobacco, maize, pea, tomato, carrot, wheat, barley, potato, soybean, sunflower, lettuce, rice, alfalfa *and beetroot*.

- the recombinant colipase or protein or derived polypeptide characterised in that it is obtained according to the invention,

- the association of recombinant pancreatic lipase and colipase or protein or derived polypeptides characterised in that it is obtained using a method of the invention,

- the enzymatically active plant extract such as obtained by using a method of the invention, characterised in that it contains recombinant pancreatic lipase and/or recombinant colipase or the proteins or the derived polypeptides,

- the pharmaceutical compositions, characterised in that they comprise an activity of pancreatic colipase type (for example human pancreatic colipase) or its derivatives, possibly associated with an activity of pancreatic lipase type (for example HPL), if required in association with a pharmaceutically acceptable vehicle,

- the method, in particular enzymatic bioconversion or biocatalysis, by implementing one or more enzymatic reactions in industrial, agro-food or agro-industrial areas, in particular the fat and lipochemistry industries and the dairy industry, these enzymatic reactions being conducted by means of plants, or parts of plants, in particular leaves and/or fruits and/or seeds and/or plant cells, genetically transformed according to the invention, having an activity of pancreatic colipase type (for example human pancreatic colipase), or of derived polypeptides possibly associated with an activity of pancreatic lipase type (for example HPL),

- the enzymatic preparations intended for industrial, agro-food or agro-industrial use, comprising one (or more) enzymatically active plant extract(s) of the invention and/or a purified or partly purified pancreatic colipase (for example human pancreatic colipase) or derived polypeptides possibly associated with an activity of pancreatic lipase type (for example HPL),

- the use of plants, or parts of plants, in particular leaves and/or fruits

and/or seeds and/or plant cells, genetically transformed according to the invention and expressing an activity of pancreatic colipase type (for example human pancreatic colipase), or of derived polypeptides possibly associated with an activity of pancreatic lipase type (for example HPL), for the implementation at industrial level of enzymatic bioconversion reactions, or biocatalyses, such as hydrolyses or enzymatic trans-esterification,

- the method of biocatalysis using plants, or parts of plants, in particular leaves and/or fruits and/or seeds and/or plant cells, genetically transformed according to the invention, said plants, or fragments, or cells, or seeds, being used both as a source of activity of pancreatic colipase type (for example human pancreatic colipase) possibly associated with an activity of pancreatic lipase type (HPL for example) and as a reaction substrate.

- use of plants, or parts of plants, in particular leaves and/or fruits and/or seeds and/or plant cells, genetically transformed according to the invention, and expressing an activity of pancreatic colipase type (for example human pancreatic lipase) possibly associated with an activity of pancreatic lipase type (HPL for example) to obtain biofuels.

- the method of obtaining a biofuel through addition of alcohol, in particular methanol or ethanol, to grindings of plants or parts of plants, genetically transformed according to the invention and/or an activity of pancreatic colipase type (for example human pancreatic colipase) possibly associated with an activity of pancreatic lipase type (HPL for example), and recovery of biofuel, in particular by filtration.

EXAMPLES

I. CONSTRUCTION OF CHIMERIC GENES ENCODING RECOMBINANT HUMAN PANCREATIC LIPASE(HPL) ALLOWING EXPRESSION IN THE LEAVES AND GRAINS OF SOLANACEAE

I.1 CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HPL AND ALLOWING EXPRESSION IN TOBACCO

The expression in the leaves and grains of tobacco of the gene coding for human pancreatic lipase (HPL) required the following regulatory sequences :

1. the double constitutive promoter 35S (Pd35S) of CaMV (Cauliflower Mosaic Virus).

This corresponds to duplication of the sequences activating transcription upstream from the TATA element of the natural 35S promoter (Kay *et al.*, 1987) ;

2. the transcription terminating sequence, polyA 35S terminator, which corresponds to the 3' non-coding region for the sequence of the circular bicatenary DNA Cauliflower Mosaic Virus producing the transcript 35S.

The constructions of the different plasmids via the use of recombinant DNA techniques derive from pBIOC4. This binary plasmid derives from pGA492 (An *et al.*, 1986) which, between the right and left borders, derived from the pTiT37 plasmid of *Agrobacterium tumefaciens*, on its transfer DNA, contains the following sequences:

the constitutive promoter of the NOS gene of nopaline synthase, the sequence encoding the nptII gene coding for neomycine phosphotransferase II deleted from the

region of the first 8 codons whose initiating codon methionine ATG is fused to the sequence of the first 14 codons of the sequence encoding the nos gene, the sequence coding for the nos gene deprived of the region of the first 14 codons, the nos terminator, a region containing multiple cloning sites (also called polylinker) (HindIII-XbaI-SacI-HpaI-KpnI-ClaI-BglII) preceding the cat gene coding for chloramphenicol, and the terminating sequences of gene 6 of the pTiA6 plasmid of *Agrobacterium tumefaciens* (Liu *et al.*, 1993). To remove almost all of the sequence encoding the cat gene, the pGA492 plasmid was doubly digested by SacI (polylinker restriction site) and by ScaI (restriction site in the cat gene sequence) then subjected to the action of the enzyme T4 DNA polymerase (New England Biolabs) in accordance with the manufacturer's instructions. Ligation of the modified plasmid (20 ng) was conducted in a 10 µl reaction medium containing 1 µl buffer T4 DNA ligase x 10 (Amersham), 2.5 U of the enzyme T4 DNA ligase (Amersham) at 14 C for 16 hours. The DH5α *Escherichia coli* bacteria, previously made competent, were transformed (Hanahan *et al.*, 1983). The plasmid DNA of the clones obtained, selected on 12 µg/ml tetracycline, was extracted using the alkaline lysis method and analysed by enzyme digestion by restriction enzymes. Then the HindIII restriction site of the plasmid DNA of the selected clone was modified to a EcoRI restriction site using a phosphorylated HindIII-EcoRI adapter (Stratagene Cloning Systems). To conduct this modification, 500 ng of plasmid DNA of the selected clone were digested by HindIII, dephosphorylated by calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) following the manufacturer's instructions and co-precipitated in the presence of 1500 ng HindIII-EcoRI DNA adapter, 1/10 volume of 3M sodium acetate, pH 4.8, and 2.5 volumes absolute ethanol at –80 C for 30 min. After centrifuging at 12000 g for 30 min., the precipitated DNA was washed in 70% ethanol, dried, recovered in 8 µl water, brought to 65 C for 10 min., then ligated in the presence of 1 µl buffer T4 DNA ligase x 10 (Amersham) and 2.5 U of the enzyme T4 DNA ligase (Amersham) at 14 C for 16 hours. After inactivation of T4 DNA ligase at 65 C for 10 min., the ligation reaction mixture was digested by EcoRI, purified by electrophoresis on O.8% agarose gel, precipitated in the presence of 1/10 volume of

3M sodium acetate, pH 4.8, and 2.5 volumes absolute ethanol at –80 C for 30 min., centrifuged at 12000 g for 30 min., washed with 70% ethanol, dried, then ligated as described above. The DH5α *Escherichia coli* bacteria previously made competent were transformed (Hanahan, 1983). The plasmid DNA of the clones obtained, selected on 12 µg/ml tetracycline, was extracted using the alkaline lysis method and analysed by enzymatic digestion by HindIII and EcoRI in particular. The resulting binary plasmid, which now only contains the last 9 codons of the sequence encoding the cat gene, with a single EcoRI site, was called pBIOC4.

The expression cassette, made up of the pd35S promoter and the polyA 35S terminator was isolated from the plasmid pJIT163Δ. The pJIT163Δ plasmid derives from the plasmid pJIT163 which itself derives from the plasmid pJIT60 (Guerineau and Mullineaux, 1993). The pJIT163 plasmid has an ATG codon between the HindIII and SalI sites of the polylinker. To remove this ATG and obtain the pJIT163Δ plasmid, the plasmid DNA pJIT163 was doubly digested by HindIII and SalI, purified by electrophoresis on 0.8% agarose gel, electroeluted, precipitated in the presence of 1/10 volume of 3M sodium acetate pH 4.8, and 2.5 volumes of absolute ethanol et – 80 C for 30 min., centrifuged at 12000 g for 30 min., washed with 70% ethanol, dried, subjected to the action of the Klenow enzyme (New England Biolabs) following the manufacturer's instructions, deproteinised by extraction with 1 volume phenol:chloroform:isoamyl alcohol (25:24:1) then 1 volume chloroform: isoamyl alcohol (24:1), precipitated in the presence of 1/10 volume of 3M sodium acetate Ph 4.8 and 2.5 volumes of absolute ethanol at –80 C for 30 min., centrifuged at 12000 g for 30 min., washed with 70% ethanol, dried and, finally, ligated in the presence of 1 µl buffer T4 DNA ligase x 10 (Amersham) and 2.5 U of the enzyme T4 DNA ligase (Amersham) at 14 C for 16 hours. The DH5α *Escherichia coli* bacteria, previously made competent, were transformed (Hanahan, 1983). The plasmid DNA of the clones obtained, selected on 50 µg/ml ampicilline was extracted using the lysis method and analysed by enzymatic digestion by restriction enzymes. To isolate the expression

cassette made up of the Pd35S promoter and polyA 35S terminator (SacI-XhoI fragment), the plasmid DNA of the selected clone pJIT163Δ was digested by SacI and XhoI. The SacI-XhoI fragment, carrying the expression cassette, was purified by electrophoresis on 0.8% agarose gel, electroeluted, precipitated in the presence of 1/10 volume 3M sodium acetate pH 4.8, and 2.5 volumes of absolute ethanol at –80 C for 30 min., centrifuged at 12000 g for 30 min., washed with 70% ethanol, dried, then subjected to the action of the Mung Bean Nuclease enzyme (New England Biolabs) according to the manufacturer's instructions. This purified insert (200 ng) was cloned in the plasmid DNA of pBIOC4 (20 ng) digested by EcoRI, treated with the Mung Bean Nuclease enzyme and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) according to the manufacturer's instructions. The ligation reaction was conducted in 20 µl in the presence of 2 µl buffer T4 DNA ligase x 10 (Amersham), 2 µl of 50% polyethylene glycol 8000 and 5 U of the enzyme T4 DNA ligase (Amersham) at 14 C for 16 hours. The DH5α *Escherichia coli* bacteria, previously made competent, were transformed (Hanahan, 1983). The plasmid DNA of the clone obtained, selected on 12 µl/ml tetracyline, was extracted using the lysis method and analysed by enzymatic digestion by restriction enzymes. The resulting plasmid was called pBIOC21.

Human pancreatic lipase (HPL) is naturally synthesised in the form of a precursor with 465 amino acids. The mature HPL protein is made up of 449 amino acids.

I.1.1 CONSTRUCTION OF THE BINARY PLASMID CONTAINING PS-HPL

The plasmid containing the complete cDNA coding for HPL was digested in order to remove the sequence encoding the signal peptide of HPL made up of 16 amino acids. This sequence was replaced by that coding for the signal peptide PS of

sporamine A of sweet potato (Murakami *et al.*, 1986 ; Matsukoa and Nakamura, 1991) with 23 amino acids (*ATG AAA GCC TTC ACA CTC GCT CTC TTC TTA GCT CTT TCC CTC TAT CTC CTG CCC AAT CCA GCC CAT TCC*) following the PCR amplification protocol described above in paragraph I.

After enzymatic digestion, the DNA fragments derived from PCR amplification were purified by electrophoresis on 2% agarose gel, electroeluted, precipitated in the presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at –80 C for 30 min., centrifuged at 12000 g for 30 min., washed with 70% ethanol, dried, then ligated to the digested plasmid DNA, purified by electrophoresis on 0.8% agarose gel, electroeluted (Sambrook *et al.*, 1989), subjected to alcohol precipitation, dried and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) following the maufacturer's instructions. Ligation was conducted using the process described above at 14 C for 16 hours. The DH5α *Escherichia coli* bacteria previously made competent were transformed (Hanahan, 1983). The plasmid DNA of the clones obtained was extracted using the alkaline lysis method and analysed by enzymatic digestion by restriction enzymes. The plasmid DNA of some selected clones was checked by sequencing with a T7 kit marketed by Pharmacia using the dideoxynucleotide method.

The PS and mature HPL sequences were cloned while maintaining their reading phases open. The cleavage sequence between the PS and mature HPL sequences is Ser-Lys. From the resulting plasmid, the fragment carrying the PS-HPL sequence was isolated by enzymatic digestion, purified by electrophoresis on 0.8% agarose gel, electroeluted, precipitated with alcohol and dried. Then this treated DNA fragment was ligated to the plasmid DNA of digested pBIOC21, treated and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) in accordance with the manufacturer's instructions. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent,

analysis of the plasmid DNA of the clones obtained and the nucleotide sequence of the fragment encoding the recombinant protein PS-HPL were conducted as described previously. The plasmid DNA of the binary vector was inserted by direct transformation in the LBA4404 strain of *Agrobacterium tumefaciens* according to the method described by Holsters *et al.* (1978).

I.1.2. CONSTRUCTION OF THE BINARY PLASMID CONTAINING PPS-HPL

The plasmid containing the complete cDNA coding for the HPL was digested in order to remove the sequence encoding the signal peptide of HPL made up of 16 amino acids. This sequence was replaced by that coding for the signal peptide PPS of sporamine A of sweet potato (Murakami *et al.*, 1986, Matsukoa and Nakamura, 1991) with 37 amino acids (*ATG AAA GCC TTC ACA CTC GCT CTC TTC TTA GCT CTT TCC CTC TAT CTC CTG CCC AAT CCA GCC CAT TCC AGG TTC AAT CCC ATC CGC CTC CCC ACC ACA CAC GAA CCC GCC*) following the PCR amplification protocol described above in paragraph 1.

The DNA fragments derived from PCR amplification were treated in similar manner to that described in **I**.1.1.

The PPS and mature HPL sequences were cloned by maintaining their reading phases open. The cleavage sequence between the two sequences is Ala-Lys. The resulting plasmid is treated as described in **I**.1.1.

The plasmid DNA of the binary vector was inserted by direct transformation in the LBA4404 strain of *Agrobacterium tumefaciens* using the method described by Holsters *et al.* (1978).

I.1.3 CONSTRUCTION OF THE BINARY PLASMID CONTAINING PSRGL-

HPL

Rabbit gastric lipase is synthesised in the form of a precursor made up of a peptide signal with 22 amino acids, located at the NH_2-terminal side and preceding the polypeptide sequence of mature lipase, the pJ0101 clone containing complete cDNA coding for rabbit gastric lipase is described in European patent application EP542629.

The plasmid containing the complete cDNA coding for HPL was digested to remove the sequence encoding the peptide signal of HPL made up of 16 amino acids. This sequence was replaced by that coding for the peptide signal of rabbit gastric lipase made up of the following 22 amino acids :

MWVLFMVAALLSALGTTHGLFG (*ATG TGG GTG CTT TTC ATG GTG GCA GCT TTG CTA TCT GCA CTT GGA ACT ACA CAT GGT CTT TTT GGA*) and following the PCR amplification protocol described above in paragraph 1.

The DNA fragments derived from PCR amplification were treated in similar manner to that described in **I**.1.1.

The PSRGL and mature HPL sequences were cloned by maintaining their reading phases open (that is to say such that they form a single open reading phase). The cleavage sequence between the PSRGL and mature HPL sequences is Gly-Lys. The resulting plasmid was treated as described in I.A.a.

The plasmid DNA of the binary vector was inserted by direct transformation into the LBA4404 strain of *Agrobacterium tumefaciens* according to the method described by Holsters *et al.* (1978).

I.1.4. CONSTRUCTION OF THE BINARY PLASMID CONTAINING PSHPL-

HPL.

The plasmid containing the complete cDNA coding for HPL (465 amino acids) comprises the peptide signal PSHPL of 16 amino acids (*ATG CTG CCA CTT TGG ACT CTT TCA CTG CTG CTG GGA GCA GTA GCA GGA*).

The DNA fragment carrying the complete cDNA coding for HPL was treated in similar manner to that described in I.1.1.

The cleavage sequence between the two sequences coding for PSHPL and HPL is Gly-Lys.

The plasmid DNA of the binary vector was inserted by direct transformation into the LBA4404 strain of *Agrobacterium tumefaciens* following the method described by Holsters *et al.* (1978).

I.2 CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HPL AND ALLOWING EXPRESSION IN TOMATO

The constructions used are the same as those cited for the genetic transformation of the tobacco plant.

II. CONSTRUCTION OF CHIMERIC GENES CODING FOR THE RECOMBINANT PROTEIN OF HUMAN PANCREATIC COLIPASE (HPCOL) AND ALLOWING EXPRESSION IN THE LEAVES AND GRAINS OF SOLANACEAE.

II.1 CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HPCOL AND ALLOWING EXPRESSION IN TOBACCO

The expression in the leaves and grains of tobacco of the gene coding for human pancreatic colipase (HPCOL) required the regulatory sequences described for human pancreatic lipase in paragraph I.1.

The binary plasmid bPIOC21 described in I.1. is also used.

Human pancreatic colipase (HPCOL) is naturally synthesised in the form of a precursor with 112 amino acids. The mature protein HPCOL is made up of 95 amino acids.

II.1.1 CONSTRUCTION OF THE BINARY PLASMID CONTAINING PSRGL-HPCOL

Rabbit gastric lipase is synthesised in the form of a precursor made up of a peptide signal of 22 amino acids, located on the NH_2-terminal side and preceding the polypeptide sequence of mature lipase; the pJO101 clone containing the complete cDNA coding for rabbit gastric lipase is described in European patent application EP542629.

The plasmid containing the complete cDNA coding for HPCOL was digested in order to eliminate the sequence coding for the peptide signal of HPCOL made up of 17 amino acids. This sequence was replaced by that coding for the signal peptide of rabbit gastric lipase made up of the following 22 amino acids :

MWVLFMVAALLSALGTTHGLFG (*ATG TGG GTG CTT TTC ATG GTG GCA GCT TTG CTA TCT GCA CTT GGA ACT ACA CAT GGT CTT TTT GGA*) following the PCR amplification protocol described above in paragraph 1.

The DNA fragments derived from PCR amplification were treated in similar

manner to that described in **I**.1.1.

The PSRGL and mature HPCOL sequences were cloned by maintaining their reading phases open (that is to say, such that they form a single open reading phase). The cleavage sequence between the PSRGL and HPCOL sequences is Gly-Ala. the resulting plasmid is treated as described in **I**.1.1.

The plasmid DNA of the binary vector was inserted by direct transformation in the LBA4404 strain of *Agrobacterium tumefaciens* according to the method described by Holsters *at al.* (1978).

II.1.2. CONSTRUCTION OF THE BINARY PLASMID CONTAINING PSHPCOL-HPCOL

The plasmid containing the complete cDNA coding for HPCOL (112 amino acids) comprises the peptide signal PSHPCOL of 17 amino acids (*ATG GAG AAG ATC CTG ATC CTC CTG CTT GTC GCC CTC TCT GTG GCC TAT GCA*).

The DNA fragment carrying the complete cDNA coding for HPCOL was treated in similar manner to that described in **I**.1.1.

The cleavage sequence between the two sequences coding for PSHPCOL and HPCOL is Ala-Lys.

The plasmid DNA of the binary vector was inserted by direct transformaiton into the LBA4404 strain of *Agrobacterium tumefaciens* following the method described by Holsters *et al.* (1978).

III CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HUMAN PANCREATIC LIPASE (HPL) AND ALLOWING EXPRESSION IN COLZA GRAINS

III.I.1. CONSTRUCTION OF THE BINARY PLASMID CONTAINING THE PCRU PROMOTER

The production of human pancreatic lipase (HPL) in colza grains required the insertion of cDNA coding for HPL between the following regulatory sequences :

1. the PCRU promoter corresponding to the 5' non-coding region for the gene of the grain reserve protein, radish CRUCIFERIN A (Depigny-This *et al.*, 1992) and allowing specific expression in the grains ;

2. the transcription terminator sequence polyA 35S which corresponds to the 3' region non-coding for the sequence of the circular bicatenary DNA of Cauliflower Mosaic Virus producing the transcript 35S.

To obtain a binary plasmid similar to pBIOC21 but whose Pd35S promoter has been replaced by the PCRU promotor, the "Klenow treated EcoRI-BamHI" fragment containing the PCRU promoter was isolated from the pBI221-CRURSP plasmid derived from pB1221 (marketed by Clontech) by replacing the 35S promoter by the PCRU promoter.

The "Klenow treated EcoRI-BamHI" fragment carrying the PCRU promoter was purified by electrophoresis on 0.8% agarose gel, electroeluted (Sambrook *et al.*, 1989), subjected to alcohol precipitation, dried and ligated to the plasmid DNA of pJIT163 (described in paragraph 1), digested by KpnI, treated with T4 DNA Polymerase (New England Biolabs) following the manufacturer's recommendations,

then digested by BamHI, purified by electrophoresis on 0.8% agarose gel, electroeluted (Sambrook *et al.*, 1989), subjected to alcohol precipitation, dried and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) in accordance with the manufacturer's instructions. Ligation was conducted with 20 ng of dephosphorylated vector described above and 200 ng of "Klenow treated EcoRI-BamHI" DNA fragments in a reaction medium of 20 µl in the presence of 2 µl buffer T4 DNA ligase x 10 (Amersham), 2 µl of 50% polyethylene glycol 8000 and 5 U of the enzyme T4 DNA ligase (Amersham) at 14 C for 16 hours. The DH5α *Escherichia coli* bacteria previously made competent were transformed (Hanahan, 1983). The plasmid DNA of the clones obtained, selected on a medium containing 50 µg/ml of ampicilline, was extracted by lysis and analysed by enzymatic digestion by restriction enzymes. The resulting plasmid was called pBIOC27.

The expression cassette, made up of the PCRU promoter and polyA 35S terminator was isolated from pBIOC27 by total XhoI digestion, followed by partial EcoRI digestion. It was purified by electrophoresis on 0.8% agarose gel, electroeluted, subjected to alcohol precipitation, dried, treated with Klenow (New England Biolabs) according to the manufacturer's instructions and ligated to the plasmid DNA of pBIOC4 at the Klenow-treated EcoRI site, and dephosphorylated with calf intestine alkaline phosphatase enzyme(Boehringer Mannheim) following the manufacturer's instructions. Ligation was conducted with 20 ng of dephosphorylated vector described above and 200 ng of XhoI-EcoRI DNA fragments described above in a 20 µl reaction medium in the presence of 2 µl buffer T4 DNA ligase x 10 (Amersham), 2 µl of 50% polyethylene glycol 8000 and 5 U of the enzyme T4 DNA ligase (Amersham) at 14 C for 16 hours. The DH5α *Escherichia coli* bacteria previously made competent were transformed (Hanahan, 1983). The plasmid DNA of the clones obtained, selected on a medium containing 12 µg/ml tetracyline, was extracted by alkaline lysis and analysed by enzymatic digestion by appropriate restriction enzymes. The resulting

plasmid was called pBIOC28.

III.1.2. CONSTRUCTION OF THE BINARY PLASMID CONTAINING PSHPL-HPL.

The fragment carrying the sequence PSHPL-HPL was ligated to the digested plasmid DNA of pBIOC28, treated and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) in accordance with the manufacturer's instructions.

Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, and the nucleotide sequence of the fragment coding for the recombinant protein PSHPL-HPL were conducted as previously. The plasmid DNA of the binary vector was incorporated by direct transformation into the LBA4404 strain of *Agrobacterium tumefaciens* using the method described by Holsters *et al.* (1978).

IV. CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HUMAN PANCREATIC COLIPASE (HPCOL) AND ALLOWING EXPRESSION IN COLZA GRAINS

IV.I.I. CONSTRUCTION OF THE BINARY PLASMID CONTAINING PSHPCOL-HPCOL UNDER CONTROL OF THE PCRU PROMOTER

The fragment carrying the PSHPCOL-HPCOL sequence was ligated to the digested plasmid DNA of pBIOC28, treated and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) in accordance with the manufacturer's instructions.

Ligation, transformation of DH5α *Escherichia coli* bacteria previously made

competent, analysis of the plasmid DNA of the clones obtained, and the nucleotide sequence of the fragment coding for the recombinant protein PSHPCOL-HPCOL were conducted as previously. The plasmid DNA of the binary vector was incorporated by direct transformation in the LBA4404 strain of *Agrobacterium tumefaciens* in accordance with the method described by Holsters *et al.* (1978).

V. CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HUMAN PANCREATIC LIPASE (HPL) AND ALLOWING EXPRESSION IN MAIZE SEEDS

V.1.1 CONSTRUCTION OF PLASMIDS CONTAINING PSHPL-HPL AND ALLOWING CONSTITUTIVE EXPRESSION IN MAIZE SEEDS.

Constitutive expression in maize seeds of the animal nucleotide sequence coding for human pancreatic lipase (HPL) required the following regulatory sequences;

1. one of the two promoters allowing constitutive expression :

- actin promoter of rice followed by the actin intron of rice (APR-AIR) contained in the plasmid pAct1-F4 described by McElroy *at al.* (1991) ;

- double constitutive promoter 35S (Pd35S) of CaMV (Cauliflower Mosaic Virus). This corresponds to duplication of the sequences activating transcription upstream from the TATA element of the natural 35S promoter (Kay *et al.*, 1987) ;

2. one of two terminators :

- the transcription terminator sequence, terminator polyA 35S, which

corresponds to the 3' non-coding region for the sequence of the circular bicatenary DNA Cauliflower Mosaic Virus producing the transcript 35S ;

- the transcription terminator sequence, terminator polyA NOS which corresponds to the 3' non-coding region for the gene of nopaline synthase of the Ti plasmid of *Agrobacterium tumefaciens* nopaline strain.

The plasmid in which the sequence coding for PSHPL-HPL is placed under the control of PAR-IAR was obtained by cloning a fragment carrying the sequence coding for PSHPL-HPL in pBSII-PAR-IAR-TNOS.

The fragment carrying the sequence coding for PSHPL-HPL was isolated by enzymatic digestion, purified by electrophoresis on 0.8% agarose gel, electroeluted, subjected to alcohol precipitation, dried, then treated. The pBSII-PAR-IAR-tNOS plasmid was digested, purified, treated and dephosphorylated by calf intestine alkaline phosphatase enzyme of calf intestine (Boehringer Mannheim) according to the manufacturer's instructions. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained and the nucleotide sequence of the fragment coding for the recombinant protein PSHPL-HPL were conducted as previously.

The pBSII-PAR-IAR-TNOS plasmid results from the cloning at the "Klenow-treated Eco01091 and KpnI" sites of pBSII-TNOS of the SnaBI-KpnI fragment carrying the sequence corresponding to "APR-AIR-start of the sequence coding for the gus gene" isolated from the pAct1-F4 plasmid. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

The pBSII-TNOS plasmid was obtained by cloning, at the dephosphorylated

EcoRV site of pBSIISK+ marketed by Stratagene, of the SacI-EcoRI fragment carrying the TNOS sequence isolated from pB1121 marketed by Clontech by double enzymatic digestion by SacI and EcoRV, subjected to purification by electrophoresis on 2% agarose gel and treated with the enzyme T4 DNA polymerase. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

The plasmid in which the sequence coding for PSHPL-HPL is placed under the control of Pd35S, was obtained by cloning at the "KpnI and BamHI" sites of the pBSII-T35S plasmid of the fragment carrying the sequence corresponding to isolated "Pd35S-PSHPL-HPL". Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

The pBSII-T35S plasmid was obtained by cloning at the Klenow-treated SpeI site, dephosphorylate of the pBSIISK+ plasmid marketed by Stratagene, of the SmaI-EcoRV fragment carrying the sequence T35S isolated from pJIT163 (described above) by double enzymatic digestion with SmaI and EcoRV, subjected to purification by electrophoresis on 2% agarose gel. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

V.1.2 CONSTRUCTION OF PLASMIDS CONTAINING PSHPL-HPL AND PSHPL-HPL-KDEL RESPECTIVELY AND ALLOWING EXPRESSION IN THE ALBUMEN OF MAIZE SEEDS.

Expression in the albumen of maize seeds of the gene coding for human pancreatic lipase (HPL) required the following regulatory sequences:

1. the promoter of the zein gene of maize (Pyzein) contained in the p63 plasmid described by Reina *et al.*, 1990. The p63 plasmid results from the cloning of Pyzein at the HindIII and Xbal sites of a pUC18 plasmid containing, between its HindIII and EcoRI sites, the expression cassette "P35S-gus-TNOS" of pB1221 marketed by Clontech. It allows expression in the albumen of maize seeds.

2. the transcription terminating sequence, polyA NOS terminator, which corresponds to the 3' non-coding region for the nopaline synthase gene of the Ti plasmid of *Agrobacterium tumefaciens*, nopaline strain.

The plasmid in which the sequence coding for PSHPL-HPL is placed under the control of Pyzein, was obtained by cloning in plasmid 63 of the fragment carrying the sequence coding for PSHPL-HPL. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

In this plasmid, the sequence coding for the tetrapeptide KDEL was incorporated upstream from the STOP codon to allow addressing in the endoplasmic reticulum.

VI. CONSTRUCTION OF CHIMERIC GENES CODING FOR RECOMBINANT HUMAN PANCREATIC COLIPASE (HPCOL) AND ALLOWING EXPRESSION IN MAIZE SEEDS.

VI.1.1. CONSTRUCTION OF PLASMIDS CONTAINING PSHPCOL-HPCOL ALLOWING CONSTITUTIVE EXPRESSION IN MAIZE SEEDS.

Constitutive expression in maize seeds of the cDNA coding for human pancreatic colipase (HPCOL) required the regulatory sequences described in V.1.1.

The plasmid in which the sequence coding for PSHPCOL-HPCOL is placed under the control of APR-AIR was obtained by cloning the fragment carrying the sequence coding for PSHPCOL-HPCOL in pBSII-APR-AIR-TNOS.

The fragment carrying the sequence coding for PSHPCOL-HPCOL was isolated by enzymatic digestion, purified by electrophoresis on 2% agarose gel, electroeluted, subjected to alcohol precipitation, dried, then treated. The pBSII-PAR-IAR-TNOS plasmid was digested, purified, treated and dephosphorylated with calf intestine alkaline phosphatase enzyme (Boehringer Mannheim) in accordance with the manufacturer's instructions. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained, and the nucleotide sequence of the fragment coding for the recombinant protein PSHPCOL-HPCOL were conducted as described previously. The pBSII-APR-AIR-TNOS plasmid used is described in V.I.I.

The plasmid in which the sequence coding for PSHPCOL-HPCOL is placed under Pd35S control was obtained by cloning, at the "KpnI and BamHI" sites of the pBSII-TS35S plasmid, the fragment carrying the sequence corresponding to isolated "Pd35S-PSHPL-HPL". Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

The pBSII-T35S plasmid was described in **V**.1.1.

VI.1.2 CONSTRUCTION OF PLASMIDS CONTAINING PSHPCOL-HPCOL AND PSHPCOL-HPCOL-KDEL RESPECTIVELY ALLOWING EXPRESSION IN THE ALBUMEN OF MAIZE SEEDS.

Expression in the albumen of corn seeds of the cDNA coding for human

pancreatic colipase (HPCOL) required the regulatory sequences described in **V**.1.2.

The plasmid in which the sequence coding for PSHPCOL-HPCOL is placed under the control of Pyzein, was obtained by cloning, in the p63 fragment, the fragment carrying the sequence coding for PSHPCOL-HPCOL. Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

In this plasmid, the sequence coding for the tetrapeptide KDEL was incorporated upstream from the STOP codon to allow addressing in the endoplasmic reticulum.

VII. CONSTRUCTION OF A BINARY PLASMID CO-EXPRESSING THE CHIMERIC GENES CODING FOR RECOMBINANT HUMAN PANCREATIC LIPASE (HPL) AND RECOMBINANT HUMAN PANCREATIC COLIPASE (HPCOL) ALLOWING EXPRESSION IN THE LEAVES AND GRAINS OF SOLANACEAE.

VII.1 CONSTRUCTION OF THE CO-EXPRESSION BINARY PLASMID pBIOC43

The co-expression binary plasmid allows expression of two genes in the same binary vector.

The co-expression binary plasmid is derived from pBIOC21. It contains two expression cassettes each made up of a Pd35S promoter and a polyA 35S terminator but which differ from each other in the polylinker separating the promoter from the terminator. One of the expression cassettes is that of pBIOC21 already described in **I**.

The other expression cassette was obtained by replacing the HindIII-BamHI-SmaI-EcoRI polylinker of pJIT163D by a HindIII-EcoRI adapter carrying the restriction sites PacI, AscI, MluI and HpaI. This adapter was obtained by renaturing the 2 oligodeoxynucleotides 5' AGC TGA TTA ATT AAG GCG CGC CAC GCG TTA AC 3' and 5' AAT TGT TAA CGC GTG GCG CGC CTT AAT TAA TC 3' which are complementary in their 28 3'terminal nucleotides. One hundred µmoles of each of these two oligodeoxynucleotides were previously phosphorylated through the action of 10U of T4 polynucleotide kinase enzyme (New England Biolabs) in a total reaction volume of 10 µl containing 1 µl of buffer T4 polynucleotide kinase x 10 (New England Biolabs) and 3 µl ATP (95mM). Both reaction mixtures were incubated at 37 C for 1 hour, then at 65 C for 20 min. They were then grouped together and their volume was completed to 500 µl. After extraction with one volume of phenol: chloroform: isoamyl alcohol (25:24:1), and 1 volume of chloroform: isoamyl alcohol (24:1), 50 µl of 3M sodium acetate pH6.0 were added. The reaction mixture was incubated at 80 C for 10 min., then slowly cooled to room temperature. The DNA was then precipitated in the presence of 2.5 volumes of absolute ethanol at –80 C for 30 min., centrifuged at 14000 g at 4 C for 1 hour, washed with 70% ethanol, centrifuged at 14000 g at 4 C for 10 min., dried, recovered in 10 µl of H20. The HindIII-EcoRI DNA fragment was then cloned to the HindIII-EcoRI sites of the plasmid DNA pJIT163D previously dephosphorylated with calf intestine alkaline phosphatase enzyme (New England Biolab) according to the manufacturer's instructions. The ligation reaction was conducted in a reaction volume of 20 µl in the presence of 1 U of T4 DNA ligase (Gibco-BRL) for a total DNA concentration of 8.5 nM with a vector/insert molar ratio of 1 and 4 µl of buffer T4 DNA ligase x 5 (Gibco-BRL) at 25 C for 16 hours. The bacteria, previously made competent, were transformed. The plasmid DNA of the clones obtained, selected on 100 µg/ml of ampicilline, was extracted using the alkaline lysis method and analysed by enzymatic digestion. The resulting clone was called pBIOC42. Its validity was checked by sequencing with the "Sequenase Version 2.0 DNA Sequencing" kit marketed by United States Biochemical (USB) using the dideoxynucleotide method. The reaction

conditions were in accordance with the manufacturer's indications except for denaturation and hybridisation. The reaction medium containing plasmid DNA (0.5 to 1 pmoles), the oligonucleotide primer (2 pmoles), 10% DMSO and the reaction buffer x 1 (USB) were incubated at 100 C for 10 min., then suddenly cooled to – 80 C on carbon ice.

From pBIOC42, the fragment coding for the expression cassette made up of the Pd35S promoter and poly 35S terminator, was isolated by double digestion by SacI and XhoI. It was purified by electrophoresis on 0.75% agarose gel, then subjected to the action of the "Geneclean II" kit marketed by BIO101 according to the manufacturer's instructions. Ligation was conducted in a reaction volume of 20 µl in the presence of 1 U of T4 DNA ligase (Gibco-BRL) for a total DNA concentration of 8.5 nM with a vector/insert molar ratio of 1 and 4 µl of buffer T4 DNA ligase x 5 (Gibco-BRL) at 25 C for 16 hours. The DH5α *E. coli* bacteria previously made competent were transformed. The plasmid DNA of the clones obtained, selected on 30 µg/ml of chloramphenicol, was extracted using the alkaline lysis method and analysed by enzymatic digestion. The resulting plasmid was called pBIOC75.

From pBIOC75, the DNA fragment carrying the expression cassette made up of the Pd35S promoter and polyA 35S terminator, was isolated by digestion with KpnI. It was purified by electrophoresis on 0.75% agarose gel then subjected to the action of the "Geneclean II" kit marketed by BIO101 in accordance with the manufacturer's instructions. Then, this fragment of DNA was ligated to the plasmid DNA of pBIOC21 digested by KnpI and dephosphorylated with calf intestine alkaline phosphatase enzyme (New England Biolabs) in accordance with the manufacturer's instructions. Ligation was conducted in a reaction volume of 20 µl in the presence of 1 U of T4 DNA ligase (Gibco-BRL) for a total DNA concentration of 8.5 nM with a vector/insert molar ratio of 1 and 4 µl of buffer T4 DNA ligase x 5 (Gibco-BRL) at 25 C for 16 hours. The DH5α *E. coli* bacteria, previously made competent, were

transformed. The plasmid DNA of the clones obtained, selected on 12 µg/ml of tetracycline, was extracted using the alkaline lysis method and analysed by enzymatic digestion. The resulting plasmid was called pBIOC43.

VII.2 CONSTRUCTION OF THE BINARY PLASMID CO-EXPRESSING THE CHIMERIC GENES CODING FOR RECOMBINANT HUMAN PANCREATIC LIPASE (HPL) AND HUMAN PANCREATIC COLIPASE (HPCOL).

The cDNAs coding for the sequences "PSHPL-HPL" and 'PSHPCOL-HPCOL" were each cloned under the control of the constitutive promoter Pd35S and the 35S terminator in the co-expression vector pBIOC43.

Ligation, transformation of DH5α *Escherichia coli* bacteria previously made competent, analysis of the plasmid DNA of the clones obtained were conducted as described previously.

VIII. EXAMPLE OF HOW TO OBTAIN TRANSGENIC COLZA PLANTS

The grains of Spring rape (*Brassica napus* cv WESTAR) or Limagrain lines) are disinfected for 40 minutes in a 15% solution of Domestos (registered trademark). After rinsing 4 times in sterile water, the grains are left to germinate, 20 grains per pot, diameter 7 cm – height 10 cm, on a mineral Murashige and Skoog medium (Sigma M 5519) with 30 g/l of sucrose and solidified with 5g/l agargel. These pots are placed in a culture chamber at 26 C with a photoperiod of 16h/8h under a light intensity of approximately 80 µE m^{-2} s^{-1}.

After 5 days' germination, the cotyledons are removed in sterile manner by cutting each petiole approximately 1 mm above the cotyledon knot.

At the same time a preculture of *Agrobacterium tumefaciens,* LBA4404 strain, containing the plasmids, namely the pGAZE plasmid, in which was inserted the sequence coding for human pancreatic lipase fused with that coding for an addressing signal (PS, PPS, PSRGL, PSPHP) under control of the PCRU promoter (or Pd35S), is made in a 50 ml Erlenmeyer vessel for 36 h at 28 C in 10 ml of 2YT bacterial medium supplemented with antibiotics appropriate for selection of the strain being used.

This preculture is used to inoculate(1%) a new bacterial culture made under the same conditions. After 14 h the culture is centrifuged for 15 min. at 3000 g and the bacteria are recovered in an equivalent volume of liquid germination medium. This suspension is divided into Petri dishes, 5 cm in diameter, to the proportion of 5 ml per dish.

The cut end of the petiole is immersed for a few seconds in a solution of agrobacteria prepared in this way, then the petiole is planted a few millimetres deep into the regeneration medium. This medium has the same basic composition as the germination medium with, in addition, 4 mg/l of benzyl-amino-purine (BAP) a phytohormone which promotes bud neoformation. Ten explants (cotyledon with petiole) are cultured per Petri dish 9 cm in diameter (Greiner reference 664102).

After 2 days' co-culture under the same environmental conditions as for germination, the explants are transplanted to phytatrays (Sigma reference P1552) containing the preceding medium supplemented with a selective agent : 45 mg/l of kanamycine sulphate (Sigma, reference K4000) and a bacteriostatic : mixture of 1/6 (by weight) of potassium salt of clavulanic acid and 5/6 sodium salt of amoxicilline (injectable Augmentin) to 600 mg/l.

The explants are transplanted twice at a 3-week interval in sterile manner on a

fresh medium under the same conditions.

The green buds which appear at the end of the second or third transplantation are separated from the explant and cultured separately in transparent pots 5 cm in diameter and 10 cm high containing an identical medium to the previous one but without BAP. After 3 week's culture the stalk of the transformed bud is cut and the bud is replanted in a pot of fresh medium. After three to four weeks, the roots have sufficiently developed to allow acclimatisation of the seedling in a phytotron. Any buds that are not green or have not taken root are removed. These seedlings are transplanted into square pots with 7 cm sides filled with compost soil (NF standard U4451: 40% brown peat: 30% sifted heath-mould: 30% sand) saturated in water. After two weeks' phytotron acclimatisation (temperature 21 C, photoperiod 16h/8h and 84% relative humidity) the seedlings are transferred to pots 12 cm in diameter filled with the same compost soil enriched with slow release Osomocote fertiliser (registered trademark) to the proportion of 4g/l of compost soil and transferred to a greenhouse (class S2) regulated at 18 C with two daily water sprinklings lasting 2 minutes.

As soon as the flowers appear they are packed in sachets (Crispac, reference SM 570y 300mm*700mm) such as to prevent cross-fertilisation.

When the siliquae reach maturity they are harvested, dried and threshed. The grains obtained are used to titre biochemical activity. Selection of transgenic descent is made by germination on a medium containing kanamycine sulphate to the proportion of 100 to 150 mg/l (according to genotype). Operating conditions are identical to those described above except that germination is conducted in glass tubes with a single grain per tube. Only those seedlings which develop secondary roots during the first three weeks are acclimatised in a phytotron before being transferred to a greenhouse.

IX EXAMPLE OF HOW TO OBTAIN TRANSGENIC SOLANACEOUS PLANTS

IX.1 OBTAINING TRANSGENIC TOBACCO PLANTS

The tobacco plants used for the transformation experiments (*Nicotiana tabacum* var. *Xanthi* NC and PBD6) are cultured *in vitro* in a Marashige and Skoog basic medium (1962) supplemented with vitamins described by Gamborg *et al.* (1968, Sigma reference M0404), 20g/l sucrose and 8 g/l agar (Merck). The pH of the medium is adjusted to 5.8 with a potassium hydroxide solution before autoclaving at 120 C for 20 min. Inter-node cuttings are taken from the tobacco plants every 30 days and planted in this MS20 proliferation medium.

All *in vitro* cultures are conducted in an air-conditioned enclosure under the following conditions :

- light intensity of 30 μ E.m^{-2},s^{-1} ; photoperiod of 16 h;

- thermoperiod of 26 C during the daytime, 24 C at night.

The transformation technique used is derived from that described by Holsters *et al.* (1985).

A preculture of *Agrobacterium tumefaciens*, LBA4404 strain, containing the plasmids is made for 48h at 28 C under stirring in an LB medium supplemented with appropriate antibiotics. The preculture is then diluted to 1/50 in the same medium and cultured under the same conditions. After one night, the culture is centrifuged (10 min., 3000g), the bacteria are recovered in an equivalent volume of liquid MS30 medium (30 g/l sucrose) and this suspension is diluted to 1/10.

Explants of approximately $1cm^2$ are cut from the leaves of the above-described seedlings. They are then placed in contact with the bacterial suspension for 1h, quickly dried on filter paper and placed on a co-culture medium (solid MS30).

After 2 days, the explants are transferred to Petri dishes on the MS30 regeneration medium, containing a selective agent, kanamycine (200 mg/l), an antibiotic, augmentin (400 mg/l) and the hormones necessary for bud induction (BAP, 1 mg/l and ANA, 0.1 mg/l). The explants are transplanted onto the same medium after 2 weeks' culture. After 2 additional weeks, the buds are transplanted into Petri dishes on a development medium made up of MS20 medium supplemented with kanamycine and augmentin. After 15 days, one half of the buds are transplanted. Root growth requires approximately 20 days after which the seedlings can be cloned by inter-node propagation or transferred to a greenhouse.

IX.2 OBTAINING TRANSGENIC TOMATO PLANTS

Tomato grains cv. UC82B are sterilised with 10% Domestos for 15min. and rinsed 3 times with sterile water. The last rinse is conducted for 10 min. under stirring.

The grains sterilised in this way are left to germinate on a MSSV/2 medium (basic medium described by Murashige and Skoog supplemented with Nitsch vitamins (Thomas and Pratt, 1981), 30 g/l sucrose, 8 g/l agar (Merck) pH 5.9 for 7 or 8 days in an air-conditioned chamber (light intensity of 30 μ E m^{-2}, s^{-1}), photoperiod of 16h/8h, 26 C).

The transformation technique used is derived from that described by Fillatti *et al.*, (1987).

A preculture of *Agrobacterium tumefaciens*, LBA4404 strain, containing the

plasmids is made for 24h at 28 C under stirring in an LB medium supplemented with suitable antibiotics. The preculture is then diluted to 1/50 in the same medium and cultured under the same conditions for one night. OD at 600 nm is measured, the agrobacteria are centrifuged (10 min., 3000g) and recovered in a liquid KCMS medium (described in the publication by Fillatti *et al.*, 1987) such as to obtain an OD at 600 nm of 0.8.

Technical improvements were made to some stages of the protocol described by Fillatti *et al.* (1987).

Preculture of explants and co-culture are conducted as described by Fillatti *et al.* (1987) except that the KCMS medium is supplemented with acetosyringone (200mM).

The 2Z washing medium differs through the addition of cefotaxime at 500 mg/l instead of carbenicilline. The development medium used is made up of the basic medium described by Murashige and Skoog (Sigma MS6899) supplemented with Nitsch vitamins, 20 g/l sucrose, 50 mg/l kanamycine, 200 mg/l augmentin, 1 mg/l ANA, 0.5 mg/l zeatin.

X. OBTAINING TRANSGENIC MAIZE PLANTS

X.1 OBTAINING MAIZE CALLI AND THEIR USE AS A TARGET FOR GENETIC TRANSFORMATION

The genetic transformation of maize, irrespective of the method used (electroporation, *Agrobacterium*, microfibres, particle gun) generally requires the use of indifferentiated fast division cells which have maintained the ability to regenerate full plants. This type of cell makes up the friable embryogenous callus (so-called type

II) of maize.

These calli are obtained from immature embryo of genotype HI II or (A188 x B73) using the method and media described by Armstrong (Maize Handbook; (1994) M. Freeling, V. Walbot Eds; pp. 665-671). The calli obtained in this way are multiplied and maintained by successive transplanting every fifteen days onto the initiation medium.

Seedlings are then regenerated from these calli by modifying the hormonal and osmotic equilibrium of the cells in accordance with the method described by Vain *et al.* (1989). These plants are then acclimatised in a greenhouse where they can be cross-bred or self-fertilised.

X.2 USE OF A PARTICLE GUN FOR THE GENETIC TRANSFORMATION OF MAIZE

The preceding paragraph describes how to obtain and regenerate the cell lines needed for transformation ; here a genetic transformation method is described leading to stable incorporation of the modified genes in the plant genome. This method is based on the use of a particle gun ; the target cells are callus fragments described in paragraph 1. These fragments with a surface area of 10 to 20 mm^2 were deposited, 4 h before bombardment, in the centre of a Petri dish, 16 fragments per dish, containing a culture medium identical to the initiation medium supplemented with 0.2 M mannitol + 0.2 M sorbitol. The plasmids carrying the genes to be incorporated are purified on a Qiagen® column following the manufacturer's instructions. They are then precipitated onto tungsten particles (M10) according to the protocol described by Klein (1987). The particles coated in this way are gun sprayed onto the target cells using the method described by J. Finer (1992).

The bombarded callus dishes are sealed with Scellofrais® then cultured in the dark at 27 C. The first transplant is made 24h later, and then every fifteen days for 3 months on an identical medium to the initiation medium supplemented with a selective agent whose type and concentration may vary according to the gene used (see paragraph 3). Suitable selective agents are generally active compounds of some herbicides (Basta®, Round-up®) or certain antibiotics (Hydromycine, Kanamycine..).

After 3 months, and sometimes earlier, calli are obtained whose growth is not inhibited by the selection agent, and are usually and chiefly made up of cells resulting from the division of a cell having incorporated into its genetic inheritance one or more copies of the selection gene. The incidence rate of obtaining such calli is approximately 0.8 callus per bombarded dish.

These calli are identified, individualised, amplified, then cultured such as to regenerate seedlings. In order to avoid any interference with non-transformed cells, all these operations are conducted on culture media containing the selective agent.

The plants regenerated in this manner are acclimatised and cultured in a greenhouse where they can be cross-bred or self-fertilised.

XI. ANALYSIS OF THE EXPRESSION OF HUMAN PANCREATIC LIPASE (HPL) IN TRANSGENIC TOBACCO PLANTS

XI.1. PROTOCOL

The protocol for extracting lipase from tobacco leaves taken from greenhouse plants is as follows : 1 g of leaves (weight when fresh) is ground in liquid nitrogen, then at 4 C in 5 ml of Tris-HCl 50mM buffer neutral pH, to which is added 1mM EDTA and 10mM β-mercaptoethanol, 0.2% Triton-X-100 and 50mM NaCl. The

entire ground mixture is immediately centrifuged at 4 C for 15 min. at 10000 g.

For tobacco grains, extraction is conducted using 0.1 g of grains per 4 ml of buffer.

Lipase activity is measured with a pH-STAT using the titrimetric method described by Gargouri *et al.* (1995) in which the substrate used is tributyrine. Tributyrine emulsion (1 ml per 30 ml emulsion) is vortexed in 2.5mM Tris-HCl buffer pH 8.0, 25mM NaCl and 5mM CaCl2. Titration consists of neutralising the butyric acid released under the action of the lipase by a soda solution at a recommended pH of 8.0 at 37 C. One lipase unit corresponds to the quantity of enzyme which causes release of one micromole of fatty acids in 1 min. at 37 C and under optimal pH conditions (8.0).

Titration of the soluble total proteins is made on the centrifugation supernatant.

An ELISA sandwich test is also made on the centrifugation supernatant with anti-natural HPL polyclonal antibodies.

XI.2 EXPRESSION WITH PLANT PEPTIDE SIGNALS ; TITRATION ON TRANSFORMED TOBACCO LEAVES AND GRAINS; ELISA TEST.

Using appropriate titration methods known to persons skilled in the art, the expression of recombinant proteins in the leaves and grains of transformed tobacco is measured.

XI.3 EXPRESSION WITH THE HPL PEPTIDE SIGNAL; TITRATION OF LIPASE ACTIVITY ON TOBACCO LEAVES.

Using appropriate titration methods known to those skilled in the art, the expression of recombinant proteins in the leaves and grains of transformed tobacco is measured.

XII. ANALYSIS OF THE EXPRESSION OF HUMAN PANCREATIC LIPASE IN TRANSGENIC TOMATO PLANTS

XII.1 PROTOCOL

The protocol for extracting lipase from the leaves and fruits of the tomato plant is similar to that described for tobacco leaves, except that 1 g of fresh product is recovered in 4 ml of buffer. Lipase activity is determined as described for tobacco leaves.

XII.2 TITRATION IN TOMATO PLANT FRUITS

Lipase activity is determined as described for tobacco leaves.

XIII ANALYSIS OF THE EXPRESSION OF HUMAN PANCREATIC COLIPASE (HPCOL) IN TRANSGENIC TOBACCO PLANTS

XIII.1 PROTOCOL

The protocol for extracting lipase from tobacco leaves taken from greenhouse plants is as follows : 1 g of leaves (fresh) is ground in liquid nitrogen, then at 4 C in 5 ml of 50mM Tris-HCl buffer pH 7.5, supplemented with 1mM EDTA and 10mM β-mercaptoethanol, 0.2% Triton X-100 and 50mM NaCl. The entire ground mixture is immediately centrifuged at 4 C for 15 min. at 10000 g.

For tobacco leaves, extraction is made from 0.1 g of grains per 4 ml of buffer.

Lipase activity is determined with a pH-STAT using the method described by Patton *et al.* (1978). The effect of the colipase on the delayed hydrolysis of the intralipids is determined as follows : the intralipids (0.5 ml) are diluted in 10 ml of a solution made up of 2mM Tris-HCl pH 8.0, 150mM NaCl, 4mM taurodeoxycholate, 1mM $CaCl_2$, at 40 C. The colipase is added, then after 1 min. the mixture is supplemented with pancreatic lipase (10^{-5})M in the proportion of 10 µl.

Colipase activity may also be measured according to the method described by Ouaged *et al.* (1982). The lipase is denatured to pH2, which enables the colipase to be preserved intact. Colipase activity is then assessed according to the preparation's ability to restore the activity of a lipase preparation lacking colipase. In practice, the sample to be tested is added to the reaction medium, acidified to pH2 with 1N HCl, and after one minute brought to pH 9 with 1M NaOH. An aliquot of semi-purified lipase of known potential activity is added. The colipase is measured according to the level of restored lipase activity from inactive lipase.

XIV. WESTERN BLOT TYPE IMMUNODETECTION OF RECOMBINANT HPL

XIV.1 HPL EXPRESSED IN THE LEAVES AND GRAINS OF TRANSGENIC TOBACCO AND COLZA

XIV.1.1 EXPRESSION WITH PLANT PEPTIDE SIGNALS ; IMMUNODETECTION IN THE LEAVES AND GRAINS OF TRANFORMED TOBACCO AND COLZA.

Western Blot type immunodetection experiments on HPL were conducted on the proteins of tobacco leaves and tobacco and colza grains extracted with the buffer

(see extraction protocol above). To conduct these experiments, the extract proteins (30 µg total proteins per sample) are first separated according to size on 12.5 % denaturing polyacrylamide gel, then transferred onto a nitrocellulose membrane. Denaturing gels may also be used (urea) and criteria other than size for sorting. An anti- human pancreatic lipase polyclonal antibody is used as a probe and development is made using a suitable anti-IgC antibody coupled to alkaline phosphatase.

The reference protein is natural human pancreatic lipase which migrates in the form of single band with an apparent molecular mass of approximately 50 kDa.

No band is detected in the protein extracts of leaves and grains of non-transformed tobacco and colza.

XIV. 1.2 EXPRESSION WITH THE HPL PEPTIDE SIGNAL ; IMMUNODETECTION IN THE LEAVES AND GRAINS OF TRANSFORMED TOBACCO.

Western Blot tests were conducted on the proteins of leaves and grains of tobacco extracted using the previously described protocol. The extracted proteins are first separated on denaturing polyacrylamide gel (SDS-PAGE) then transferred onto a nitrocellulose membrane. An anti-HPL polyclonal antibody is used as probe and development is made using a suitable antibody coupled to alkaline phosphatase.

The reference protein is human pancreatic lipase which migrates in the from of a single band with an apparent molecular mass of approximately 50 kDa. No band is detected in the protein extracts of non-transformed tobacco leaves. (T).

XIV. 1.3 HPL IN AN EXTRACT CONTAINING PROTEINS OF TRANSFORMED TOBACCO LEAVES. DEGLYCOSYLATION TEST.

The protein extraction protocol for deglycosylation tests is as follows : 0.5 g of leaves (weight when fresh) are ground in liquid nitrogen, then at 4 C in 1 ml of denaturation buffer (100mM phosphate buffer pH 7.5), supplemented with 1% β-mercaptoethanol, 25mM EDTA and 1% SDS. The crushed mixture is centrifuged at 4 C for 15 min. at 10000 g. The supernatant is incubated for 5 min. at 100 C to achieve denaturation of the proteins, then centrifuged 2 min. at 10000 g. The supernatant is then diluted to 1/10 in the deglycosylation buffer (100mM phosphate buffer pH7.5), supplemented with 1% β-mercaptoethanol, 25mM EDTA, 0.1% SDS and 1% octylglucoside). The enzyme (N-glycosidase F, PN Gase Boehringer) is added to the proportion of 1 U per 100 µl of supernatant. A reference with no enzyme is used for each sample.

Deglycosylation of the reference protein (human pancreatic lipase) takes place under the same conditions. The different protein samples are incubated at room temperature for 8 hours. The proteins are then separated by electrophoresis on polyacrylamide gel and transferred onto a nitrocellulose membrane as described in the preceding paragraph.

XV. WESTERN BLOT TYPE IMMUNODETECTION OF RECOMBINANT HPCOL.

Western Blot type immunodetection assays (Renart and Sandoval, 1984) of HPCOL were conducted on the proteins of tobacco leaves and tobacco and colza grains extracted with the buffer (see extraction protocol above). To conduct these tests, the extracted proteins (30 µg total proteins per sample) are first separated on denaturing polyacrylamide gel using the Tris-tricine method described by Okajima *et al.* (1993), then transferred onto a nitrocellulose membrane. An anti- human pancreatic lipase polyclonal antibody is used as a probe and development is conducted using a suitable anti-IgG antibody coupled to alkaline phosphatase.

XVI. PURIFICATION OF HUMAN PANCREATIC LIPASE FROM PLANTS

XVI.1 PURIFICATION OF HPL FROM TOBACCO LEAVES

Recombinant human pancreatic lipase is purified by ion-exchange chromatography on a DEAE-Fast Flow column (Pharmacia) equilibrated in 50mM Tris-acetate buffer pH 7.0. The column is washed with the same buffer until absorption at 280 nm is less than OD 0.05. The fixed enzymatic activity is eluted with a linear salt gradient in the same buffer (0 to 0.5M NaCl) using 5 volumes of column. The fractions containing the enzymatic activity are grouped together. They are subjected to filtration through Sephadex G-100.

XVII. PURIFICATION OF HUMAN PANCREATIC COLIPASE FROM PLANTS.

The protein is purified using conventional protein purification methods known to those skilled in the art.

XVIII. SYNTHESIS OF FATTY ACID ESTERS

The tests were conducted with non-transformed colza grains, lipase being provided :

- either in the form of an immobilised enzyme preparation (lipozyme (NOVO) or free (human pancreatic lipase).

- or in the form of transformed tobacco grains with the gene of human pancreatic lipase.

Esterification reactions are conducted at 37 C for 16 hours in airtight glass bottles placed on a stirring table (250 rpm). The organic solvent used is hexane in which the fatty acids are soluble. Methanol is added in stoichiometric proportion to the theoretical quantity of triacylglycerol contained in the colza grains.

The major component of the fatty acids of colza is oleic acid, therefore the reference sample chosen is a methyl ester of oleic acid. Synthesis follow-up is made by thin layer chromatography (TLC). The migration solvent is a mixture of hexane, diethylether, water (70:10:1). Plate development is made immediately after spraying 5% sulphuric acid in ethanol.

Use of the recombinant pancreatic lipase of the invention leads to obtaining the expected product which is methyl ester of oleic acid.

XIX. TEST FOR HUMAN PANCREATIC LIPASE ACTIVITY

The activity test is conducted using Lipase-PS reagents, Sigma Dianostics, (Method n 805)9 according to the manufacturer's instructions. Procedure is based on the colorimetric method of Imamura *et al.* (Clin. Chem. 35:1126,1989). The 1.2 diglyceride is hydrolysed into 2-monoglyceride and fatty acids. The 2-monoglyceride is then measured by coupled enzymatic reaction catalysed by monoglyceride lipase, glycerol kinase, glycerophosphate oxydase and peroxydase. It is a highly sensitive, specific test for pancreatic lipase due to the use of colipase and deoxycholate as activators.

Measurements were taken on the following samples :

- 2 protein extracts of tobacco leaves transformed with LBA4404 comprising the binary plasmid containing PSHPL-HPL. The plants were analysed 3

weeks after greenhouse transfer.

- 1 protein extract of non-transformed tobacco leaf (negative reference),

- 1 protein extract of non-transformed tobacco leaf mixed with purified human pancreatic lipase marketed by Sigma (reference : L9780) at 500 U/I (reconstruction).

The values obtained were the following :

- 270 U/I for the negative reference

- 874 U/I for the reconstruction

- 1113 U/I for one of the samples

- 1572 U/I for the other sample.

These results reveal the presence of lipase activity in both samples.

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